IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Patent Application of: Arthur SCHAFFER

Serial No. : 10/069,389

Filed : June 27, 2002

For : METHOD FOR BREEDING TOMATOES HAVING REDUCED WATER CONTENT AND PRODUCT OF THE METHOD

Group Art Unit: 1638

Examiner: Keith O. Robinson

RULE 132 DECLARATION OF ARTHUR SCHAFFER

I, the undersigned, Arthur Schaffer, of 16 Hazayit Street, Hashmonaim, Israel hereby declare as follows:

1. I am the Applicant in U.S. Patent Application Serial No. 10/069,389, filed June 27, 2002 (hereinafter "the application").

2. I received a Ph.D. in Plant Genetics and Physiology from Rutgers University in 1982 and was a post-doctoral Fellow at the Hebrew University, Faculty of Agriculture, Rehovot, Israel from 1982-1985. Since 1985, I have served as a Research Scientist at ARO, The Volcani Center, Institute of Field and Garden Crops. I have served as Chairman of the Department of Vegetable Crops at ARO since 2000. I have co-authored many articles that have been published in *Plant Physiology*, *Plant Science* and other publications in the field of plant physiology.

3. The claims of the aforesaid application, and specifically claims 1 – 16,

stand rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement.

4. Specifically, the Examiner notes that claims 1 - 4 and 6 - 16 are broadly drawn to a method for breeding tomato plants that produce tomatoes with reduced fruit water content, comprised of crossing *Lycopersicon esculentum* with <u>any other</u> *Lycopersicon* species (Emphasis added). The Examiner further notes that the specification only provides guidance for crossing a single *Lycopersicon* species, namely *L. hirsutum*, with *L. esculentum*

5. It is respectfully submitted that the teaching in the specification of crossing a single *Lycopersicon* species, namely *L.hirsutum*, with a cultivated *L. esculentum* is enabling for other wild *Lycopersicon* species. Evidence of this may be seen in research conducted by the Applicant and a collaborator, which is described in Israel Patent Application Serial No. 164125, a copy of which is attached hereto as Appendix A.

6. Appendix A describes the cloning of the gene determining the trait of fruit dehydration and shows that the gene (termed by us as PUT, for putative protein) is expressed in developing fruit of tomatoes carrying the *L. hirsutum* derived allele for the gene and thereby exhibiting the genetic trait of dehydration, but is not expressed in genotypes of tomato carrying the *L. esculentum* allele for the PUT gene and thereby lacking the genetic trait of dehydration. The application also shows that the gene derived from other wild species besides *L. hirsutum* is similarly expressed when introgressed into the *L. esculentum* background. As stated in Appendix A, pages 12-13, "In addition, we examined the expression of the PUT gene in another NIL (near inbred line) population, the introgression line 4.4 derived from the interspecific hybridization of *L. esculentum* (M82) and an additional wild species *L. pennellii*, containing the analogous introgression as the *L. hirsutum*-derived genotypes described here (Eshed and Zamir, 1994). This population represents another wild allele of the PUT gene, and the fruit of IL4.4 also show micro-fissures and dehydrate. Similar to the *L. hirsutum* derived populations, the *L. pennellii* derived introgression containing the *L. pennelii* allele for *Cwp* (IL 4.4) showed expression of the PUT gene in the young fruitlets, compared to M82".

7. In addition, a recently published article (A. Frary, T.M. Fulton, D. Zamir and S.D. Tanksley, 2004, Theoretical and Applied Genetics vol. 108:485-496) a copy of which is attached hereto as Appendix B, notes that the recently described genetic trait of epidermal reticulation (*er*) is observable in *L. esculentum* introgression lines derived from additional wild species, including *L. parviflorum* and *L. peruvianum* (pages 490-491).

8. Additionally, Appendix A shows that the trait of fruit dehydration is related to the development of microfissures in the fruit cuticle (page 6). As these fissures or cracks may at times be suberized they presumably lead to the trait of epidermal reticulation (*er*), or "melon like" skin.

9. Accordingly, the fact that the genetic composition of other wild species of *Lycopersicon*, such as *L. parviflorum and L. peruvianum*, can lead to the undesirable trait of epidermal reticulation as shown in Appendix B, indicates that these other wild species of *Lycopersicon* also can be used as sources for the trait of cuticle cracks, or microfissures, leading to the trait of fruit dehydration.

10. Beginning at page 2, last two lines of the Official Action, the Examiner states that it appears that reduced water content is correlated with or identical to increased soluble solids content.

11. The claims, as amended, all include a recitation of fruit dehydration producing a reduction in red ripe fruit water content of at least 30%, as distinguished from lowered water content which is a corollary of increased soluble solids content, such lowering being substantially less than 30% and not involving dehydration of existing ripe fruit water.

12. Accordingly, while breeding for increased soluble solids content may be unpredictable and species dependent, breeding for fruit dehydration is predictable based on the teachings of the present application. Accordingly, the plant, which is the subject of claims 15 and 16, as amended, is readily obtainable by a repeatable method set forth in the specification. For this reason, a deposit is not required.

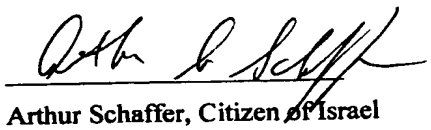
13. Beginning at page 6 of the Official Action, the Examiner rejects claims 1 - 16 under 35 U.S.C. 112, first paragraph as failing to comply with the written description requirement. At page 7 of the Official Action, the Examiner notes that the Court in University of California v Eli Lilly and Co. 43 USPQ2d 1398, 1406 (Fed Cir. 1997) held that to adequately describe a claimed genus, the Patent Owner must describe a representative number of the species of the claimed genus and that one of skill in the art should be able to "visualize or recognize the identity of the members of the genus".

14. Example 1 at page 5, line 31 of the specification refers explicitly to Accession Number LA 1777, which is deposited and classified as a representative *L. hirsutum*, as evidenced by the classification in the Report of the Tomato Genetics Cooperative (Revised List of Wild Species Stocks, by R. T. Chetelat, Report of the Tomato Genetics Cooperative, vol. 54 (2004) pg. 62), a copy of which is attached hereto as Appendix C. The stocks of *Lycopersicon* wild species are held at the C.M. Rick Tomato Genetics Resource Center, Department of Vegetable Crops, University of California, Davis, CA 95616. Accordingly, it is respectfully submitted that one of skill in the art should be able to "visualize or recognize the identity of the members of the genus".

15. A prior art publication, (The New Rural Industries, a Handbook for Farmers and Investors, ed. Keith Hyde, published Dec. 1997, pg. 230) attached hereto as Appendix D, describes *Solanum centrale*, an aboriginal species of a native desert "bush tomato" whose fruits turn from green to yellow and which is left on the bush to dry and shrivel and resembles a raisin. It is respectfully submitted that although referred to colloquially as a "bush tomato", *Solanum centrale* is not a tomato and is not hybridizable with the *Lycopersion esculentum* and thus does not adversely affect the patentability of claims 1 - 14.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and conjecture are thought to be true; and further that these statements are made with the knowledge that willful false statements so made are punishable by fine or imprisonment, or both, under Section 1001 of Title

18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.



Arthur Schaffer, Citizen of Israel
16 Hazayit Street, Hashmonaim, Israel
November 30, 2003

Appendix A

A GENE CONTROLLING FISSURE DEVELOPMENT IN TOMATO FRUIT CUTICLES

Inventors: Arthur Schaffer and Ran Hovav

Abstract of the Disclosure

Cultivated tomatoes (*Lycopersicon esculentum*) have evolved a fruit cuticle that is relatively impervious to water and therefore the whole fruit cannot dehydrate. We have developed a tomato containing an allele derived from the wild species of *Lycopersicon hirsutum* which is characterized by a permeable fruit cuticle and thereby the ability to naturally dehydrate as a whole fruit. We have identified the gene controlling the trait and have developed transgenic tomato plants expressing the gene. In particular, we have accomplished the following:

1. identified the inheritance pattern of the trait of fruit dehydration derived from *L. hirsutum* as a single major gene which we suggest be termed *Cwp (Cuticular water permeability)*;
2. mapped, cloned and sequenced the gene and identified it as encoding for a putative protein (PUT) with no known biological function, as yet;
3. showed that the wild species allele for PUT allows for expression of the gene in developing tomato fruit while the standard cultivated *L. esculentum* allele is not expressed and may be considered a null allele;
4. showed that there is an allele dosage effect at the expression level and the heterozygous HE genotype is characterized by approximately half the expression as the homozygous genotype with two alleles from the wild species;
5. produced transgenic tomato plants expressing the PUT gene in order to show the effect of the gene *in planta.*

This gene may be used in tomatoes and other fruit with either overexpression of the gene or for the cessation of expression of the gene, thereby controlling fruit water loss.

Introduction

Aerial portions of higher plants are covered with a continuous extracellular layer of cuticle. The cuticle is a polymer matrix which is mostly composed of cutin monomers (primarily short-chain hydroxylated fatty acids) and various amounts of cuticular waxes (solvent-soluble lipids). Both the cutin and the wax components vary greatly in amount and composition between different plant species and plant organs (Holloway, 1982). Although the components and structure of plant cuticle as well as the biological and genetic regulation of its biosynthesis has been extensively investigated (Kolattukudy, 1980; Koornneef *et* al., 1989; Blee and Schuber, 1993; Arts *et* al., 1996; Negruk *et* al., 1996; Millar *et* al., 1997; Todd *et* al., 1999; Yaphremov *et* al., 1999; Flebig *et* al., 2000; Pruitt *et* al., 2000; Wellesen *et* al., 2001 Hooker *et* al., 2002; Chen *et* al., 2003; Kuns and Samuels, 2003; Kurata *et* al., 2003; Aharoni *et* al., 2004; Schnurr *et* at. 2004;), the mechanisms controlling the differentiation and/or function of the cuticle remain to be characterized.

The tomato fruit cuticle is a thin layer with a 4-10 micron thickness with two unique structural properties (Wilson and Sterling, 1976). First, the cutin deposits are arranged in a laminar structure – the layers are assembled in parallel to the epidermis cells. The second property of the tomato fruit cuticle is that it has not contain any stomata, pores or channels. As a result, the water permeability of the tomato skin is very low and the fully ripe tomato fruit retains its water content.,

The water permeability of a number of other cuticles lacking stomata (astomatous) and the mechanism of water transport across them have been the subjects of numerous investigations (Schönherr, 1976a; Schönherr and Schmidt, 1979; Riederer and Schreiber, 2001). Apparently, both the cutin and wax components have an integrated effect against water loss from the organ. In some cases, the thickness of the cuticular layer is inversely proportional to diffusion of water across cuticular membranes (Lownds et al., 1993). However, frequently the cuticular wax component is primary in affecting plant water permeability. For example, removal of the epicuticular wax layer from tomato fruit cuticles by organic solvents increased their water permeability by a factor of 300 to 500, as evidenced by rapid plant dehydration (Schönherr, 1976b). Additional evidence for the role of cuticular waxes as a transpiration barrier in tomato fruits is the recently reported

gene encoding the enzyme very-long-chain-fatty acid (VLFA) β-ketoacyl-CoA synthase (*LeCER6*, Vogg *et* al., 2004). This gene plays an important role in the synthesis of VFLA which are a major component in fruit cuticular wax. A loss of function mutation in this gene led to the reduction of n-alkanes and aldehydes with chain lengths beyond C_{30} in both leaf and fruit waxes. Tomato fruits with the *LeCER6* mutation were characterized with a 4- fold increase in water permeability.

Another factor affecting water permeability of tomato fruit cuticle is the presence of cracking on the cuticular surface. Fruit cracking has received much research attention (Cotner et al., 1969; Voisey et al., 1970; peet, 1992; peet and willits, 1995). Tomato fruits are affected by three main types of cracking: 1) Concentric cracking (coarse cracking); 2) Radial cracking (splitting); and 3) Cuticle cracking (russeting) (Bakker, 1988). The first two types of cracking are deep and extended fissures that penetrate through the fruit pericarp and in addition to water loss also allow pathogen penetration and fruit decomposition.

Unlike radial or concentric cracks, cuticle cracks are superficial micro fissures of the cuticle that are generally confined to the cuticle and do not penetrate to the pericarp cells.. The causes and circumstances leading to fruit cracking in tomatoes are mostly unclear and may be related to cuticular layer thickness (Emmons and Scott, 1998), shape of the underlying epidermis cells (Conter *et al.*, 1969; Emmons and Scott, 1998), fruit shape (Considine and Brown, 1981), fruit size (Koske *et al.*, 1980; Emmons and Scott, 1997), relative humidity around the fruit (Young, 1947; Tukey, 1959), strong foliage pruning (Ehret et al., 1993) and the tensile strength and extensibility of the epidermis (Bakker, 1988).

The occurrence of cracks in tomato fruit also has a significant genetic component, which is mainly expressed upon gene transfer from wild species of *Lycopersicon*. Fulton et al. (2000) described a trait, "Epidermal reticulation" (*Er*), and, using an advanced backcross QTL analysis strategy (with the wild type *L. parviflorum*) reported four QTLs affecting it. Cuticlar cracks also have been reported in *Lycopersicon* fruit derived from crosses of *L. esculentum* and other wild species such has *L. hirsutum* (WO 0113708, METHOD FOR BREEDING TOMATOES HAVING REDUCED WATER CONTENT AND PRODUCT OF THE METHOD)) and *L. penellii* (Monforte et al., 2001).

Cracks in fruit cuticle, particularly extreme cracks which are visually evidenced as epidermal relticulation, due to the development of a suberized coating

along the fissure (Monforte et al., 2001), are generally considered to be negative phenomenon due to the esthetic damages that lower fruit value (Tukey, 1959), as well as due to the loss of fruit moisture content. However, the economic potential of fruits that dehydrate while whole and while still attached to the vine, is high. Dehydrated tomato products comprise an important portion of the tomato industry. The production of tomato pastes, ketchup, and other processed tomato products is dependant on the energy-requiring steps of dehydration. In addition, "sun-dried" tomato fruit are prepared in a drying process which consists of dehydrating cut tomato fruit either in the sun or in drying ovens. Both sun-drying and oven drying may lead to losses in food quality. For example, levels of ascorbic acid, one of the major nutritional contributions of tomatoes in the human diet, decrease significantly in response to sun-drying or oven-drying (Ojimelukwe, 1994). Furthermore, the necessity to cut the tomato fruit in half before the drying process does not allow for the production of whole dried tomato fruit.

In this work, we used a map-based positional cloning strategy to clone a gene from the wild species *L. hirsutum* that increases the cuticular water permeability (CWP) of the mature red tomato fruit and leads to the dehydration of the intact fruit. We suggest *Cwp* as the term for the novel gene. *Cwp* encodes for a protein (putative protein) with no known biological function. This gene may contribute to breeding programs for new tomato products, as well as for other crops, as it controls water loss through the cuticle. Furthermore, the structural phenotype of micro-fissures associated with this gene indicates a role for *Cwp* in fruit cuticle development. Thus, it may illuminate the genetic regulation of plant cuticle development, in general, and the tomato developing fruit cuticle, in particular.

Materials and methods

Plant material and measurements

A set of near – isogenic introgression lines derived from a backcross breeding program based on the inter-specific hybridization of *L. esculentum* (E) and the wild species *L. hirsutum* (H), distinguished by the trait of fruit dehydration was developed, as described previously (WO 0113708, METHOD FOR BREEDING TOMATOES HAVING REDUCED WATER CONTENT AND PRODUCT OF THE METHOD) and will be summarized here. Plants of E breeding line 1630 were pollinated with wild

species H (LA1777). Hybrid F_1 plants were self-pollinated, generating F_2 seeds. Three F_2 plant were selected based on their high sugars content when ripe. F_3 seeds were sown and ten plants of each of the F_3 plants of these three F_2 selections were grown, and fruit was allowed to remain on the vine past the normal stage of ripening and harvest. Among the F_3 plants one plant (F3-203-10) showed the characteristic of sign of fruit dehydration, evidenced by wrinkling of fruit skin. A pedigree breeding program was developed consisted of selfing this F_3 individual until the F_4 generation followed by intense selection for fruit dehydrating rate. Plants then were backcrossed to the E breeding line, with the product of this cross being selfed for four additional generations to produce a BC1F4 population. Dehydrating individuals from this population were subjected to another backcross to E, producing hybrid plants that were presence with the trait. Two F_2 populations (2394 and 2395) were constructed from these F_1 individuals.

Initially the selection procedure was based on the phenotype of fruit dehydration and microcracks on the fruit cuticle. Following the development of molecular markers linked to the trait, selection was performed according to the genotype. Cleaved Amplified Polimorphic (CAPS) marker were used as the molecular markers. CAPS were developed using a specific PCR product that was cut by an endonucleases enzymes (for more details see at "DNA Analysis" in this chapter).

Plants were grown in 15-1 pots in a greenhouse, according to standard methods, as previously described (Miron and Schaffer, 1991). Fruits mean weight and dehydration rate were determined by picking and weighing five mature red fruits from each plant, placing them on a net-table at room temperature (appr. 25°c) and weighing them every 2-3 days. The presence of microcracks on the fruit cuticle was verified by either magnifying glass (20X ?) or binocular microscope (50X ?).

DNA analyses

Genomic DNA was extracted according to Folton *et* al. (1995). CAPS (Cleaved Amplified Polymorphism) markers were developed from RFLP markers selected from high-density tomato map (Tanksley et al. 1992), as follows. BlueScript plasmid vectors (Stratagene) containing tomato DNA inserts representing the selected RFLP markers were kindly provided by the Tomato Genome Center in Weizmann Institute of Science, Rehovot, Israel. Genomic DNA insertion segments were partially sequenced at the DNA Analysis Unit in the Hebrew University, Jerusalem, Israel,

using T7 and SP6 primers. According to these sequence analysis results sequence-specific PCR primers were designed using the Primer Express Program, version 1.0 (Perkin Elmer Biosystems). A total of approximately 20 markers were designed and these were tested to determine the existence of polymorphisms between the *L. esculentum* and *L. hirsutum* parental genotypes as well as between the tomato lines differeing in the *L. hirsutum*-derived trait.

Here we demonstrate the PCR primers for two markers – TG163 and TG587, representing positions on chromosome 4:

TG163 F: 5'-TGCAATCCCGAACATGAAGAC-3'
TG163 R: 5'-CCTTCTGGTCGCATCTGTGTCT-3'
TG587 F: 5'-TCAGGGTGAGGGGTAATAATTGAG-3'
TG587 F: 5'-GCTTAAAACTCAAGTCTCCTCGCA-3'

The amplification reactions were performed in an automated thermocycler (Mastercycle Gradient, Eppendorf, Germany) using thermostable Taq DNA polymerase (SuperNova Taq Polymerase, JMR Products, Kent, UK). The reactions were carried out in 25ul final volume that contained 10 x reaction buffer, 0.125 mM of each deoxynucleotide, 0.5 micrograms of each primer, 2.5 Unit of Taq polymerase and 50-100 nanograms of tomato genomic DNA. The conditions were optimized for the annealing temperature for each set of primers and the product fragment size. To identify restriction endonucleases that would generate a polymorphism between the *L. esculentum* and *L. hirsutum* alleles, reaction were carried out in 10ul final volume containing 3.5 microlitter of PCR product, 1 microlitter of 10 x concentrated restriction enzyme buffer, and 1-3 unit of the appropriate restriction endonuclease. The digestion products were analyzed on 1 % gels. *DraI* and *HinF1* were found to be appropriate for TG163 and TG587, respectively, and were used on the segregating populations. A similar procedure was applied for the design of the others CAPS markers.

All BACS (Bacterial Artificial Chromosomes) that were used in this work were provided from Clemson University Genomic Institute (Clemson, North Carolina, USA), using the Tomato Heinz 1706 BAC Library Filters (LE_HBa). Tomato BAC library filters were screened for a specific BAC clone by a radioactive probe, that was labeled using the NEBlot^{TM} Kit (New England BioLabs inc. #N1500S) and according

to the supplier's instructions. Labeled BAC colonies on the filter were detected using a phosphor-imager device (FLA-5000; FujiFilm). BAC plasmids were purified from the matching E. coli strains using the QIAGEN® Maxi Plasmid Purification Kit (#12263). For "Chromosome Walking" procedure, BACs ends were sequenced using the SP6 and T7 primers and a PCR product was developed according to the BACs end sequence. The new purified PCR product was radioactive labeled and was used for another round of tomato filter colonies detection.

LE_HBa 37B8 BAC clone (Clemson University Genomic Institute, Clemson, North Carolina USA) was sub-cloned into the BlueScript II ks+ vector (Stratagene) and sequenced. The 15 kb section was completely sequenced by developing primers and cloning by PCR and sequencing the relevant sections, as described above. DNA sequences were analyzed using the NCBI nucleic acid and translated protein databases by using the BLAST software (Altschul *et* al., 1990).

RNA and Quantitative RT-PCR analyses

For the preparation of cDNA, total RNA was extracted, as previously described (Miron *et* al, 2002). Total RNA was used as a template for first strand cDNA synthesis with the Super-script II pre-amplification system reverse transcriptase kit (Gibco BRL, LifeTechnologies, UK) at 42°C according to the supplier's instructions.

PCR primers. Specific primers with short amplicons for on-line quantitative PCR were designed with the Primer Express Program, version 1.0 (Perkin Elmer Biosystems) based on the sequences derived from the BAC sequencing of the three ORFs: 1) ZINC gene, forward, 5'-AATAATGCGAATCGAATCACTA-3' and reverse, 5'-AAGGCTAAATCTCCTCCTTTCT-3' (amplicon 140 bp). 2) DBP gene, forward, 5'-TGGATAAGCGGACGACTCTATTG-3' and reverse, 5'-CTGTTGTTTGGGAAGTGGCTTCT-3' (amplicon 180 bp). 3) PUT gene, forward, 5'-CTCTCCTTGGCCCAAGGCTCAA-3' and reverse, 5'-CAGCTTTAGTGGTATCTCTCATCA-3' (amplicon 130bp). Actin was used as a reference gene, with the following primers, based on Gene bank accession No. BF096262: forward, 5'-CACCATTGGGTCTGAGCGAT-3' and reverse, 5'-GGGCGACAACCTTGATCTTC-3' (amplicon 251bp).

The cDNA was used as template for quantitative PCR amplification on the GeneAmp 5700 Sequence Detection System (PE Biosystems) using SYBR Green

Master Mix containing AmpliTaq Gold, According to manufacture's instructions (PE Biosystems). The thermocycler was programmed for 40 cycles for all reactions, with the first step of denaturation at 95°C for 30 sec, the annealing temperature of 62°C for 15 sec, and extension temperature of 72 °C for 30 sec. Data acquisition was done at 77°C for 30 sec. Preliminary dissociation analyses of the PCR products showed that product remaining above 77°C was the specific PCR product. Standard curves containing logarithmically increasing known cDNA levels were run with each set of primers, in addition to the actin primers for normalization. All real time PCR products were tested on 2% agarose gel and were sent for sequencing for identity approval.

Transgenic plants

Cloning procedures. Full length sequence of the putative protein gene (*put*) was amplified from cDNA that was extracted from HH line fruit (10 days after anthesis), using the following primers: Put forward, 5'-GTAGTACTATATAAACCATGTGAG-3' and reverse, 5'-CATATGTTGACATATCTAATG-3'. The full length gene (930 bp) was cloned to pGEM-T easy vector (promega) using T-A cloning procedure, and then was sub-cloned to BlueScript II ks+ vector (Stratagene) using the *EcorI* (NEB #R0101) endonuclease. The *put* gene was again sub-cloned between the cauliflower 35S promoter and the n-terminator sites of the pBIN PLUS binary vector (Ghosh *et* al., 2002) using the *XhoI* (NEB #R0146) and *XbaI* (NEB #R0145) endonucleases. Constructed vector were transformed into *E. coli* (strain DH5alpha, Stratagene), and then were retransformed into EHA105 *Agrobacterium* electro-competent cells using the method described by Walkerpeach and Velten (1994). Plasmids were prepared using a mini-prep kit (Qiagen # 12143) and re-transformed to pBIN PLUS for sequencing to insure the absence of deletions and other cloning inaccuracies.

Transformation. Tomato transformation experiments were carried out using the cv MicroTom as described by Meissner et al. (1997) and cv. MP1 as described by Barg et al. (1997). Transgenic shoots were rooted on Murashige and Skoog basal medium (Duchefa, Haarlem, The Netherlands) supplemented with 1 mg L^{-1} zeatin (Duchefa #Z0917), 100 mg L^{-1} kanamycin and 100 mg L^{-1} Chlaforan. Standard practices of growing the transformed plants are carried out.

Results

Inheritance Analysis

The inheritance of the trait of appearance of micro-fissures on the fruit skin was determined in two independent segregating F_5 populations (lines 2394 and 2395) based on a cross between a standard small fruited cultivar (line 1815) and an advanced introgression line exhibiting the trait of dehydration (line 1881). The distribution pattern of the appearance of micro-fissures in the segregating populations was according to a ratio of 3:1 for Micro-fissured: standard cuticle, with chi-square probability values of 0.515 and 0.862 for 2394 and 2395 populations, respectively (**Table 1**). This distribution pattern is characteristic for a single gene inheritance with dominant/recessive allelic relations.

The trait of fruit dehydration (CWP) segregated according to a 3:1 ratio in population 2394 while in population 2395 segregation was according to a 1:2:1 ratio with approximately half of the population dehydrating but at an intermediate rate of dehydration. Therefore, we conclude that the allelic relations are either completely dominant or semi-dominant, depending on the genetic background of the population (**Fig.**). Therefore, we conclude that the trait of fruit CWP is inherited as a single gene trait, which we term *Cwp*.

Fine mapping of *cwp* gene

Based on the high-density tomato RFLP map (Tanksley *et* al. 1992) a set of CAPS (Cleaved amplified polymorphism) markers were designed. Loci representing various genomic positions, including markers linked to QTLs for reticulated epidermis (Fulton, *et* al., 2000) (markers TG464, TG477, CT68 and TG68 localized on chromosomes 4, 6, 8, 12, respectively) were investigated for analysis of linkage with the trait of micro-fissures.. Each polymorphic PCR-based molecular marker was applied to both parents and a set of 48 F_2 individuals segregating for the trait.

Based on the initial set of markers the *Cwp* gene was mapped to the telomeric portion of chromosome 4, linked to CT199 marker by an estimated distance of approximately 3 cM (2 recombination events in 96 gametes) (**Fig. 2A**). For finer mapping of the telomeric portion of chromosome 4 an additional group of CAPS markers were designed for a cluster of markers located throughout this chromosomal segment. The chromosomal introgression segment from the *L. hirsutum* parent was

localized between the CT163 and TG464 markers (**Fig. 2B**). This introgression represents the *L. hirsutum* segment in the near-isogenic line that was used as the dehydrating donor parent in this analysis.

In order to further narrow down the introgression size a larger F_2 population (over 200 individuals) was investigated with PCR-based markers between CT199 and TG464 markers. A closely linked cluster (<1.5 cM) of molecular markers was defined as flanking the *Cwp* gene (**Fig. 2C**) and based on this study the *Cwp* gene was located between TG464 and CT61 (0.5 cM).

Positional Cloning of cwp gene

The localization to this small introgression allowed for the positional cloning of *Cwp*. For this purpose an additional 3500 segregating progeny (7000 gametes) of a heterozygous individual derived from the near–isogenic line were subjected to CAPS marker analysis with the marker TG464 and CT61, revealing 12 recombinants (0.34 cM compared with 0.5 cM between the same markers in the "first round" of fine mapping). A set of 5 contiguous BACs bridging the linked markers TG464 and CT61 was identified and assembled using the chromosome walking technique. In brief, this was accomplished by sequencing the BAC end and using the BAC end as a probe to identify a contiguous BAC. In order to place the new BAC with respect to the introgression, and to produce a higher resolution map polymorphic CAPs for the two species were developed and the recombinants were tested for these new markers.. The 5 contiguous BACs created a bridge between CT61 and TG464 CAPS markers (**Fig. 4A**). For each of the 12 recombinant plants 10 selfed progenies were grown, genotyped with the appropriate segregating markers and analysed for dehydration and the appearance of micro-fissures. Of the 12 recombination events initially identified, 3 were further localized between the two ends of BAC 37B8 (**Fig. 4A** - area restricted by two broken lines) indicating that *Cwp* was located in the 37B8 BAC. To further resolve the recombination events, BAC 37B8 was sub-cloned and the smaller fragments were assembled in order and a segment of approximately 15,000 bp (15 kb) was identified, within which the *Cwp* gene was located. (**Fig. 4B**, mapping and sub-cloned contigs data at a lower resolution are not presented).

Bioinformatical analysis of the candidate genes

The segment of 15 kb was sub-cloned into the Bluescript vectors (Stratagene), sequenced and assembled using the SEQUENCHER software package (Gene Codes Corporation).

A bioinformatics analysis of the 15 kb sequence after analysis by the BLAST program (NCBI, http://www.ncbi.nlm.nih.gov) revealed three candidate open reading frames (ORFs) **(Fig. 5).** The first ORF showed a similarity to a protein of unknown function from *Arabidopsis thaliana* (NP_189369.1) (protein Identity - 44%, Homology - 61%). This protein has two domains. The first one is RING-finger domain (rpsBLAST – NCBI Conserved Domain Search), a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, and is probably involved in mediating protein-protein interactions (Borden, 1998). It was identified in proteins with a wide range of functions, such as viral replication, signal transduction, and development. It has two variants, the C3HC4-type and a C3H2C3-type (RING-H2 finger), which have different cysteine/histidine pattern. The other domain is DUF23 and it is domain of unknown function. It is part of a family that consists of an approximately 300 residue long region found in *C. elegans* and drosophila proteins. This region contains several conserved cysteine residues and several charged amino acids that may function as catalytic residues. We termed this ORF "Zinc". Interestingly, the homology of the tomato Zinc to the Arabidopsis homolog is not at the site of the "Ring finger" but only at DUF23 one and the "Ring finger" domain region is missing at Zinc tomato gene.

The second ORF showed similarity to a DNA-binding bromodomain-containing protein (Arabidopsis thaliana NP_974153.1|) (protein identity - 37%, Homology 56%). This gene is a part of a DNA binding protein family that is associated with acetylation regulation of proteins, DNA and chromatin and are part of histone acetyltransferase regulation (Dhalluin *et* al., 2000). We termed this gene "DBP" (DNA Binding Protein).

The third ORF had similarity to a protein described merely as an "expressed protein" (Arabidopsis thaliana NP_568038.1) (protein Identity - 48%, homology – 67%). It contains a domain of unknown function (DUF833). It is part of a family that is found in eukaryotes, prokaryotes and viruses and has no known function. One member has been found to be expressed during early embryogenesis in mice (Halford *et* al., 1993). We termed this gene as "PUT" (putative). None of these three candidate

genes showed any similarity or homology to genes that participate in known steps of cuticle biosynthesis metabolism.

Expression analysis of the candidate genes

In order to determine which of the three candidate genes is associated with tomato fruit cuticle development we measured the expression level of each of the three genes in the near-isogenic lines differing in their *Cwp* allele *(L. hirsutum* dehydrating allele, (HH), and *L. esculentum* not dehydrating allele, (EE)) (**Fig. 6**). mRNA from ovaries and fruits of the following stages was extracted: anthesis, 5 and 15 days after anthesis, and at immature green, mature green and breaker developmental stages. Fruit specimens were taken from the same segregating population that was used for the positional cloning procedure. We examined the expression of each of the genes by RT-PCR. DBP was expressed only at the ovary stage and equally in both genotypes (HH and EE) thereby indicating that the expression of this gene is not associated with the phenotypic trait (**Fig. 6B**). Expression of the Zinc gene was not observed at any fruit stage in either genotype, similarly indicating that its expression is not associated with the trait of dehydration.

Only *PUT* was expressed in the young stages of the developing fruit and, furthermore, was expressed differentially only in fruit of the dehydrating genotypes with the *L. hirsutum* allele for *Cwp* (HH) (**Fig. 6A**). The highest expression observed in this study was at the fruitlet stage of 15 days after anthesis.

In order to confirm the differential expression pattern of the PUT gene, we analyzed the expression of this gene in additional populations derived from the M82 tomato industry cultivar. One population was an F_2 population derived from a heterozygote individual, originating from the hybridization of a dehydrating line (line 2168) with the M82 determinate cultivar. We examined the expression of all three segregating genotypes (HH, HE, EE), at the stage of 5-15 days after anthesis (the stage with the highest expression levels in the first expression analysis). As shown at **Fig. 7**, a classical Mendelian expression pattern of PUT gene was found, with the HH genotypes showing highest expression levels, the heterozygous HE individuals showing approximately half the expression level, and the EE genotypes lacking expression.

In addition, we examined the expression of the PUT gene in another NIL population, the introgression line 4.4 derived from the interspecific hybridization of *L.*

esculntum (M82) and an additional wild species *L. pennellii,* containing the analogous introgression as the *L. hirsutum*-derived genotypes described here (Eshed and Zamir,1994). This population represents another wild allele of the PUT gene, and the fruit of IL4.4 also show micro-fissures and dehydrate. Similar to the *L. hirsutum* derived populations, the *L. penellii* derived introgression containing the *L. pennelii* allele for *Cwp* (IL 4.4) showed expression of the PUT gene in the young fruitlets, compared to M82 (**Fig. 7**).

Transgenic tomato plants expressing the PUT gene

In order to show that the expression of the Put gene is associated with the unique cuticular development trait transgenic tomato plants were developed with the PUT gene under the control of the 35S promoter. The phenotypic trait is observed in the transgenic plants, indicating that the expression of Put is associated with the trait.

Discussion

Cultivated tomatoes (*Lycopersicon esculentum*) have evolved a fruit cuticle that is relatively impervious to water and therefore the whole fruit cannot dehydrate. We have developed a tomato containing an allele derived from the wild species of *Lycopersicon hirsutum* which is characterized by a permeable fruit cuticle and thereby the ability to naturally dehydrate as a whole fruit. We have identified the gene controlling the trait and have developed transgenic tomato plants expressing the gene. In particular, we have accomplished the following:

1. identified the inheritance pattern of the trait of fruit dehydration derived from *L. hirsutum* as a single major gene which we suggest be termed *Cwp (Cuticular water permeability)*;
2. mapped, cloned and sequenced the gene and identified it as encoding for a putative protein (PUT) with no known biological function, as yet;
3. showed that the wild species allele for PUT allows for expression of the gene in developing tomato fruit while the standard cultivated *L. esculentum* allele is not expressed and may be considered a null allele;
4. showed that there is an allele dosage effect at the expression level and the heterozygous HE genotype is characterized by approximately half

the expression as the homozygous genotype with two alleles from the wild species;

5. produced transgenic tomato plants expressing the PUT gene in order to show the effect of the gene *in planta*.
6. This gene may be used in tomatoes and other fruit with either overexpression of the gene or for the cessation of expression of the gene, thereby controlling fruit water loss.

It is likely that the genetic trait of a relatively impervious fruit cuticle was a positive development in the evolution and domestication process of cultivated tomatoes, allowing for the stability of the ripening and harvested fruit. The genetic control of the trait of dehydration indicates a selection procedure for the null *Cwp* at some stage of evolution and domestication of the crop.

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Figure Legends:

Fig. 1. Effect of Cwp genotype on dehydration rate in population (A) and population 2149 (B). In the population 2148 the trait of dehydration behaves as a completely dominant trait while in 2149 it behaves as a partially dominant trait. Fruit were picked when red-ripe and allowed to dehydrate at ambient room temperature and weighed at approximately daily intervals. Data are expressed as Log % weight.

Fig. 2: Fine mapping of *CWP* gene: **A**: CAPS marker analysis of the TG464 molecular marker. Genomic DNA was extracted from 20 F_2 individuals segregating for dehydration rate. PCR analysis was performed using the appropriate primers for TG464 marker which showed polymorphism between the two parental species. PCR products were cleaved with *HinF1* endonuclease restriction site enzyme, and electrophorosed on 2% agarose gel. The + or – signs indicate the presence or absence of microfissures and the dehydrating condition. E – *L. esculentum*. H - *L. hirsutum*. .M – *HindIII/EcorI* lambda marker (Fermentas #SM0191).**B**. Genetic linkage map (in cM) of the chromosomal region of *CWP* oriented relative to the position of the centromere. *Lycopersicon peneellii* introgration lines IL4.3 and IL4.4 (Eshed and Zamir, 1995) are indicated. The hatched bar represents the *L. hirsutum* segment in the near-isogenic line that was used as the dehydrating donor parent in this analysis. **C.** Magnification of the chromosomal segment flanking the *Cwp* gene.

Fig. 3: Physical positioning of *CWP* gene. A. Genetically ordered contiguous BACs creating a bridge between CT61 and TG464 CAPS markers, and phenotypic analysis of the recombinants and the characterization of the recombinants according to polymorphisms of the sequenced BAC ends. Each recombinant genotype is represented by a bar divided into hatches (*L. hirsutum* genotype) and empty (*L. esculentum* genotype) segments. B. Magnification of the three crossover events in BAC 37B8. The three crossover events are those of the first three recombinants presented in Fig, 3A.

Fig. 4: Representation of the 15 kb introgression from *L. hirsutum* which includes the *Cwp* gene. The sequence was analyzed for homologous open reading frames using the NCBI program TBLAST. Three homologous sequences were identified and the direction of the open reading frame id indicated by arrows.

Fig. 5: Expression analysis of the PUT (A) and the DBP (B) genes in developing ovaries and fruitlets of tomato. Expression was measured on extracted cDNA as described in the Methods section using an On-line quantitative PCR and is expressed relative to the expression of the actin gene in each sample. Ov, ovary; 5 and 15 days after anthesis; IG, immature green, MG, mature green; B, breaker stage. Hatched bars are the Cwp^{HH} genotypes and solid bar is the Cwp^{EE} genotypes.

Fig. 6: Expression analysis of the PUT gene in 15 day fruitlets of tomato genotypes. HH, Cwp^{HH} genotype; HE, heterozygous Cwp^{HE} genotype; EE, Cwp^{EE} genotype. The three genotypes were selected from a segregating heterozygous population. IL4.4 represents the *L. pennellii* introgression line IL4.4 (Eshed and Zamir, 1985) which contains the *L. pennellii* homologue of PUT. M82 is the recurrent *L. esculentum* parent of the IL 4.4.

SEQUENCES

Genomic DNA (L. Hirsutum)

```
1       TGCCGTCCTA TTCTTAGAAT ACTCAAGTAA TTTAACGTAG TGGTGAAAAT TTGATAAATT
61      AATTATATAC TAATTTTTCA GTCTTATTTT ATGTGGTATA TTTAATTGGA TATGTAGTTT
121     AAGAAATAAT AAAAACTTTA AAATATTTAT AAATTTACTT TTCTAAAAAA GTGAATTCAA
181     TTTTTTCTCT CCTCATAAAT GTATTAGAGT ATTATCATTA AAATTAAGTG GGACTAATAA
241     AGGTAAAAAA TAAATTATTC CTTTAAATTA TTTAACCATA TAAGAAAATG TGACATTCTT
301     TTTTAGACTT GACTAAAATA GAAAATAATG TCATATATAT AAAATGAGAC GAAAAAAGTA
361     AATATTAATT TAAAATTTAA AACTTTAGGG TAATAGCTAC TTTGAATTAC CTAGATTTCA
421     ATAAAATTCA ACATATAATA AAACATACTA ATTTACAATT TTTAAAATAA TATGACTAAA
481     AGTCATATTA TTCAAAAAAC AATCTATACC GCCGTCACCT AGTTACTTTA ATTTGTGTAG
541     CTTCTAGTAC ATACATTTTT AAACTTTATC TGAATTTAAT ATTTTAATTA TATTAAACAT
601     TTATTAAAAT TTATAAAATT TAAATTGACG TAATATAATG AAGAGAGTAG TACTATATAA
661     ACCATGTGAG TACTAACATG ATCTTCATCT TATTCTTGTT TTTATTTATA GAAACAATAA
721     AATAGTTATA AAATTAATCA ATCATGTGTA TAGTAGTGTT TATTTGGGAA GCAGATAGTA
781     GATATTCATT AGTGTTATTA TTGAATAGAG ATGAATATCA TAATAGGCCA ACAAAGGAAG
841     TTCATTGGTG GGAAGATGGA GAAATTGTTG GTGGCAAAGA TGAAGTTGGT GGTGGCACTT
901     GGTTGGCTTC TTCAACTAAT GGTAAATGGC TTTCTTACTA ATGTTTTGGA ACTTCAGACA
961     CTTCCTCATG CCAAAACTAG AGGTGACCTA CCTGTTCGAT TTTTACAGGT ACGATTAAAT
1021    TCTTTATATA TTATACGTTA ATATGTTTGA TCTTTCATTT TGGTTTTGTT ATACGAAGGA
1081    CGAGACCTAG AGGTCTTTAA GACAAAACAT AAATATGCAT CATAGTCATA AACTTTCAAT
1141    AAATATTCAA TTTTGAATAT GCGCTTTCAA AGGTATTACA AGTTGAGTAC TAAAGGAATT
1201    GAGTTTATCA AGATTAAATT TTGAATTTGA TTCTTTTGAT CATGATTAAT AGTAATGTTA
1261    AATCTTGTCC TTATTGGAGT ATATATATGA TCAATAAATC AAGATTTTAA ATTGTAGTAT
1321    AATCTTAATT TTAAAGAATA TTAATGTTGT AAAATTTTAG ATTTAACAAA CACAAAAATC
1381    ATATTTGTAT GTTATAACTA TAGTTTGTAT AGTTGCGCTC AATATGTTTG TTCGCGAGCT
1441    GTTAATATGT CACTATTTCG GTTTACATAT ACAAAAGAGA TCAATTGCAT AATTTTGTTT
1501    NGCATATACN TNTTAAACAT GATACATAAT AGAAATTTCA TTNATTGTGT AATATATCTT
1561    TGTATAAAGC AAGAAAGAGC GAAACACAAC AGAAAACTGG ATAGGGAAAT ATTTATATTT
1621    TGTATAGTTA TAAGTGTATA TGACGGAAAT ATACGTAATT ATTTTTTATA CATGATTTTC
1681    TCTCGCTTTT ATGCAAACAC AAACACAATT TATACATTTG TTTTTGTGTA AAGTGAGAGT
1741    GGCGAGCGAG ATTCTATAGA GAGAGAACCA AATGAAAATA TATGTATTAT ATGCAGTTTT
1801    CTGTAGTTTT ATACAAATAC AAACACAATT TATACATTTA TTTTTGTGTA TGAGAGAGGC
1861    GAGTGAGATT CTCNGGGGAG GAAAATATAT GTATATATAC AGTTTTGTTT CGCTATAAAC
1921    AAACAGAACA CATTTTATAC ATTTGTATTT GTATAAAACA AGAGAGACGA GGGAGAAACT
1981    GCTCAACGAG AAATTCAGGA AGAGAGGTGA ATGACAACTA TTTGTTACGA GTTGCAAGTA
2041    AATCAAACTG CGACTATAAC ATTTAGTTTG AATTAATAAT TTGTTATTTT AAACGATTTT
2101    CCGTAAAATT TAATTGTTAA TTGCAGAGCA ATAAAAGCCC AATGGAGTTT GCAAAAGAGT
2161    TGGTGAATGA AGGGAATGAA TACAATGGGT TTAATTTAAT TTTGGCAGAT ATTGAAACTA
2221    AAAAAATGGT ATATGTAACA AATAGGTCCA AAGGAGAGCC CATAACAATA CAAGAAGTCC
2281    AACCAGGTAT TCATGTGCTG TCCAATGCAA AACTGGACTC TCCTTGGCCC AAGGTAAGAA
2341    TTCTAATGGG CTTTTTTCGA TCGATATACA TAAATTATAC AAATGATATG CTTTTGGTTG
2401    TTCATTTCAG GCTCAAAGAC TGAAGTTAAA TTTTAAGAAA ATGTTGGATG TTTACGAAGT
2461    GAATGACGAG AAAATCTTGG TCAAAGATAT GATAGAAAAA TTGATGAGAG ATACCACTAA
2521    AGCTGATAAA AGTAAATTGC CTTGTATTTG TTCTACAGAC TGGGAGTTGG AACTTAGCTC
2581    TATTTTCGTG GAAGTTGACA CTGCACTGGG TAATTCATAC CGCGTTATAA CTAATATGTT
2641    TGTTTGATTT TAACGTACTC AAACGATGAT AAAGGTTAAA GTAGATATAC AAACATTTTA
2701    AAAATAATTG AAATAGTTCA ATAATAGAAG TGTACATATC ATTAACATAG TTTGATGGGT
2761    TTTTTTGGTG GTGTGAATAT GTAGGGGTGT TATGGTACTA GAAGTACAAC AGCATTGACA
2821    ATTGAAGTGC GAGGAGAAGT AAGCTTTTAT GAGTTGTACC TTGAGAACAA CATGTGGAAA
2881    GAGCAAATTG TCAACTATCG GATTGAAAAA GTCCAAATGC AATAAATGTC TTTAATATGT
2941    TGATATATCT AATGTTTTTC ATGTTCATAT GTTGACATAT CTAATGTTTT CATTTTTTTT
3001    TTTTAATTCA AATAAGATTT TTTCTTCAAA AAATTAAACT TTTTGTCTTT GAATGGAAAT
3061    TGTTATTCAT TGTATTTGTA AAATGTACTA CACTACTTGG AAGACATAAT GTATGTTTCG
3121    GGCTCCTTTG TTTTAGCAAC AATTTTAGAC TTTCA
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5UTR: 679-743
Exon1: 744-1008
Intron1: 1009-2126
Exon2: 2127-2334
Intron2: 2335-2411
Exon3: 2412-2607
Intron3: 2608-2683

Exon4: 2684-2922
Stop codon, 2933-2935

cDNA Sequence (L. Hirsutum)

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 1      TGATCTTCAT CTTATTCTTG TTTTTATTTA TAGAAACAAT AAAATATTTA TAATCAATCA
61      TCATTTGTAT AGTAGTGTTT ATTTGGGAAG CAGATAGTAG ATATTCATTA GTGTTATTAT
121     TGAATAGAGA TGAATATCAT AATAGGCCAA CAAAGGAAGT TCATTGGTGG GAAGATGGAG
181     AAATTGTTGG TGGCAAAGAT GAAGTTGGTG GTGGCACTTG GTTGGCTTCT TCAACTAATG
241     GTAAATTGGC TTTTCTTACT AATGTTTTGG AACTTCATAC ACTTCCTCAT GTCAAAACTA
301     GAGGTGACCT ACCTCTTCGA TTTTTACAGA GCAATAAAAG CCCAATGGAG TTTGCAAAAG
361     AGTTGGTGAA TGAAGGGAAT GAATACAATG GGTTTAATTT AATTTTGGCA GATATTGAAA
421     CTAAAAAAAT GGTATATGTA ACAAATAGGC CCAAAGGAGA GCCCATAACA ATACAAGAAG
481     TCCAACCAGG TATTCATGTG CTGTCCAATG CAAAACTGGA CTCTCCTTGG CCCAAGGCTC
541     AAAGACTGAA GTTAAATTTT AAGAAAATGT TGGATGTTTA CGAAGTGAAT GACGAGAAAA
601     TCTGCGTCAA AGATATGATA GAAAAATTGA TGAGAGATAC CACTAAAGCT GATAAAAGTA
661     AATTGCCTTG TATTTGTTCT ACAGACTGGG AGTTGGAACT TAGCTCTATT TTCGTGGAAG
721     TTGACACTGC ACTGGGGTGT TATGGTACTA GAAGTACAAC AGCATTGACA ATTGAAGTGG
781     GAGGAGAAGT AAGCTTTTAT GAGTTGTACC TTGAGAACAA CATGTGGAAA GAGCAAATTG
841     TCAACTATCG GATTGAAAAA CTCCAAATGC AATAAATGTT TTTAATATGT TGATATATCT
901     AATGTTTTCA TG
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Initiation and stop codons are underlined.

Protein sequence:

```
1       MCIVVFIWEA DSRYSLVLLL NRDEYHNRPT KEVHWWEDGE IVGGKDEVGG GTWLASSTNG
61      KLAFLTNVLE LHTLPHVKTR GDLPLRFLQS NKSPMEFAKE LVNEGNEYNG FNLILADIET
121     KKMVYVTNRP KGEPITIQEV QPGIHVLSNA KLDSPWPKAQ RLKLNFKKML DVYEVNDEKI
181     CVKDMIEKLM RDTTKADKSK LPCICSTDWE LELSSIFVEV DTALGCYGTR STTALTIEVG
241     GEVSFYELYL ENNMWKEQIV NYRIEKLQMQ
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Claims:

1. A cultivated tomato (*Lycopersicon esculentum*) plant and/or fruit characterized by a permeable fruit cuticle and hence capable of naturally dehydrating as a whole fruit.
2. The cultivated tomato plant and/or fruit of claim 1, being transformed with any of the polynucleotides described herein.
3. A cultivated tomato (*Lycopersicon esculentum*) plant and/or fruit , being transformed with any of the polynucleotides described herein.
4. An isolated polynucleotide as described herein and functional homologs and portions thereof.
5. An isolated polypeptide as described herein and functional homologs and portions thereof.
6. A method of producing a dried fruit by overexpressing any of the polynucleotides and/or functional homologs and portions thereof in the fruit.
7. A method of transforming a fruit into a non or slow drying fruit by inhibiting the endogenous expression or activity of the *Cwp (Cuticular water permeability)* gene thereof.
8. An isolated polynucleotide encoding a polypeptide having a cuticular water permeability modulation activity.
9. A plant transformed to express a polynucleotide encoding a polypeptide having a cuticular water permeability modulation activity.

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Appendix B

ORIGINAL PAPER

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Advanced backcross QTL analysis of a *Lycopersicon esculentum* × *L. pennellii* cross and identification of possible orthologs in the Solanaceae

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Abstract In this study, the advanced backcross QTL (AB-QTL) mapping strategy was used to identify loci for yield, processing and fruit quality traits in a population derived from the interspecific cross *Lycopersicon esculentum* E6203 × *Lycopersicon pennellii* accession LA1657. A total of 175 BC_2 plants were genotyped with 150 molecular markers and BC_2F_1 plots were grown and phenotyped for 25 traits in three locations in Israel and California, U.S.A. A total of 84 different QTLs were identified, 45% of which have been possibly identified in other wild-species-derived populations of tomato. Moreover, three fruit-weight/size and shape QTLs (*fsz2b.1*, *fw3.1*/*fsz3.1* and *fs8.1*) appear to have putative orthologs in the related solanaceous species, pepper and eggplant. For the 23 traits for which allelic effects could be deemed as favorable or unfavorable, 26% of the identified loci had *L. pennellii* alleles that enhanced the performance of the elite parent. Alleles that could be targeted for further introgression into cultivated tomato were also identified.

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Introduction

Twenty years ago the first molecular genetic-linkage map of tomato was published (Tanksley et al. 1992). This map was based on an F_2 population derived from an interspecific cross between cultivated tomato, *Lycopersicon esculentum*, and its wild relative, *Lycopersicon pennellii*. Since this initial report, maps for other and more advanced *L. esculentum* × *L. pennellii* populations (for example, Eshed and Zamir 1995; Haanstra et al. 1999) and for populations from other wild species crosses (for example, Goldman et al. 1995; Tanksley et al. 1996; Fulton et al. 1997a; Bernacchi et al. 1998) have been published. Frequently these interspecific populations have also been used for the identification of quantitative trait loci (QTLs) for important agronomic and horticultural traits. As a result, comprehensive QTL information is now available for populations derived from several wild species of tomato: *Lycopersicon hirsutum* (Bernacchi and Tanksley 1997; Bernacchi et al. 1998), *Lycopersicon peruvianum* (Fulton et al. 1997b), *Lycopersicon parviflorum* (Fulton et al. 2000) and *Lycopersicon pimpinellifolium* (Grandillo and Tanksley 1996; Tanksley et al. 1996; Doganlar et al. 2002a). Most of this information was provided by analysis of advanced backcross (AB) populations. Although two studies examined some growth (de Vicente and Tanksley 1993) and yield-related (Eshed and Zamir 1995) parameters in *L. pennellii*-derived F_2 and introgression-line populations, there has been no report of QTLs identified in a *L. pennellii* AB population.

The AB-QTL mapping strategy integrates the processes of QTL discovery and introgression from wild germplasm into elite material (Tanksley and Nelson 1996). Instead of an F_2 population, this approach uses BC_2 or BC_3 populations derived from an interspecific cross for the identification and mapping of trait loci. Thus, both molecular-marker and phenotypic analyses occur at a more advanced generation when the cultivated parent's alleles are at a much higher frequency. Once favorable alleles for various loci are identified, only a few more crosses are required to develop near-isogenic lines

that can be field-tested and used for variety development. The AB-QTL method was first applied in tomato (Tanksley et al. 1996) and has since been adapted for use in rice (Xiao et al. 1996, 1998; Moncada et al. 2001), wheat (Huang et al. 2003), maize (Ho et al. 2003) and pepper (Rao et al. 2003).

The present paper describes the results from an AB-QTL study of a *L. esculentum* (cultivar E6203) × *L. pennellii* (accession LA1657) BC_2/BC_2F_1 population. *L. pennellii* is found in some of the most-arid habitats of all tomato species, and accessions within the species can exhibit extreme genetic variability (Rick and Tanksley 1981). Like most other *L. pennellii* accessions, LA1657 is self-incompatible. Both of the previous *L. pennellii* QTL studies used the self-compatible accession, LA716 (de Vicente and Tanksley 1993; Eshed and Zamir 1995). Both LA1657 and LA716 are from the western regions of Peru; however, their distributions are not identical. LA1657 is usually found in northern regions of the geographic distribution while LA716 is found in southern regions. Moreover, LA1657 prefers higher elevations (about 700 m) than LA716 (20 m) (Rick and Tanksley 1981). Because LA1657 is from a different region of the geographical distribution of the species and is genetically divergent from LA716, it was chosen for this study. The AB-QTL population was grown in three locations in two important tomato-producing regions: Israel and California, U.S.A. Plots were assessed for 25 yield, processing and fruit-appearance traits. Thus, this work extends tomato AB-QTL analyses to a fifth wild species and allows more extensive cross-species comparisons of the control of agronomically important traits in tomato and other solanaceous crops.

Materials and methods

Population development and field evaluations

The population was developed using the processing inbred line *L. esculentum* cultivar E6203 (hereafter referred to as LE) as the recurrent parent and *L. pennellii* accession LA1657 (hereafter referred to as PN) as the donor parent. A total of 320 BC_1 plants were derived from a single F_1 individual and were genotyped with several RFLP markers to select against undesirable phenotypes. TG125 was used to select for homozygous LE alleles at the self-incompatibility locus, *S*, on chromosome 1 to increase the fertility of the plants. TG167 and TG36 were used to screen for LE alleles at fruit-weight QTLs on chromosomes 2 (*fw2.2*) and 11 (*fw11.3*), respectively, to select for larger fruit. In addition, TG279 was used to select for homozygous LE alleles at the *Sp* locus on chromosome 6, thus ensuring that the plants would have a determinate growth habit. This type of growth habit is essential for mechanical harvesting of processing tomatoes. After this marker-assisted selection, eight BC_1 plants were backcrossed to LE to obtain 175 BC_2 plants which were genotyped with RFLP markers for map development. BC_2F_1 families were derived from each of the BC_2 individuals by crossing with TA496 (E6203+*Tm2*^{a}) and were field-tested during the summer of 1998 in Akko, Israel (IS), Woodland, California, U.S.A. (CA1) and Acampo, California, U.S.A (CA2). Plants were grown in randomized plots of 30 plants each with six plots of LE as controls.

Marker and linkage analysis

Genomic DNA extraction, restriction enzyme digestion, Southern hybridization, washing and autoradiography were performed as described in Bernatzky and Tanksley (1986). Parental DNA was surveyed for polymorphism after digestion with *Eco*RI and *Hin*dIII using RFLP markers that were selected at 3-cM intervals from the high-density tomato map (Tanksley et al. 1992). From the surveys, 150 polymorphic markers spanning the entire genome at intervals of less than 20 cM were chosen to genotype the BC_2 individuals.

Marker segregation was tested for significant ($P<0.001$) deviation from the expected frequency of heterozygotes for a BC_2 population (25%) using the χ^2 goodness-of-fit analysis. The "group" and "ripple" commands of Mapmaker (Lander et al. 1987) were used to establish the most-likely order of markers in each linkage group at LODs 4.0 and 3.0, respectively. Recombination was computed in Kosambi units (Kosambi 1944) using the QGene computer program (Nelson 1997).

Trait evaluations

A total of 25 agronomic traits were evaluated for each plot. Six of the traits were measured at all three locations, seven at two locations and the remaining 12 at only one location. The criteria used for assessing each trait are described below.

Yield traits

Total yield (YLD), red yield (RDY) and percent green yield (PGY) were measured in both IS and CA1. YLD was measured in kilograms and pounds, respectively, and included both ripe (red) and unripe (green) fruit. RDY was the weight of the ripe-red fruit and the weight of the unripe fruit was used to calculate PGY. Plant fertility (FERT) was evaluated only in CA2 using a scale of 1 to 5. A low-fertility rating indicated that the plot had reduced fruit set while a high rating indicated heavy fruit set. The percentage of rotten fruit on the plants in a plot (ROT) at harvest time was assessed only in IS.

Processing traits

Soluble solids content (SSC) was measured in all three locations in Brix using a refractometer as described in Tanksley et al. (1996). Higher values indicated increased sugar content. Soluble solids content was multiplied by red yield to obtain Brix × red yield (BRY) in IS and CA1. This value gives an estimate of the amount of processed product that can be expected from a given line. Juice viscosity (VIS) was measured as Bostwick only in CA1, lower values indicated greater viscosity. Fruit pH (PH) was also measured only in CA1. Thickness of the fruit pericarp (PCP) was evaluated on transverse sections of the fruit on a scale of 1 to 5 (1, thin; 5, thick pericarp) in IS and CA2, and in millimeters in CA1. Fruit firmness (FIR) was assessed by hand-squeezing the fruit (1, soft; 5, very firm). Stem retention (STR) was evaluated only in IS as the percentage of fruit that retained their stems after harvest by shaking the fruit from the plants.

Fruit appearance

Fruit weight (FW) in grams was measured on a random sample of approximately 50 fruit from each plot in IS and CA1. In CA2, fruit size (FSZ) was rated visually (1, very small; 5, very large). Fruit shape (FS) was also measured visually in all three locations on a scale of 1 to 5 where 1 indicated round fruit and 5 indicated elongated fruit.

Fruit color was assessed in four ways. The external color (EC) of ripe fruit was measured using a scale of 1 to 5 (1, light-red; 5, dark-red) in all three locations. Internal color (IC) was also

measured on transverse sections of the fruit in all locations using the same scale as EC. The amount of orange coloration (OR) on the fruit exterior was measured only in CA2 using a scale of 1 to 5:1, very orange; 5, very red. Fruit color (FC) was also measured on raw, de-aerated puree using a spectrophotometer in CA1.

Puffiness (PUF), or the amount of intralocular air space in transversely cut fruit, was evaluated in IS and CA1 using a scale of 1 to 5 (1, very puffy; 5, not puffy). Epidermal reticulation (ER) was measured in IS and CA2, and described whether the fruit skin was smooth (scored as 1) or reticulated like a cantaloupe (scored as 5). The percentage of the fruit that were cracked (PCF) was evaluated only at CA1. Yellow eye (YE) assessed whether the stem-scar penetrated into the fruit. This was measured in CA1 by examining longitudinally cut fruit and estimating the percentage of fruit with YE. Grey wall (GW) was measured in CA1 on transversely cut fruit and was also assessed as a percentage of the fruit with GW. The color of the gel (GG) in the interior of the fruit was scored in CA2 using a scale of 1 (green-gel) to 2 (red-gel).

Data analysis

Pearson's correlation coefficients were calculated for each trait/location combination using the QGene program (Nelson 1997). QGene was also used to perform single-point regression analysis to identify molecular markers with significant linkage to each trait. A QTL is only reported here if it was observed in two or more locations at $P<0.01$ or in one location at $P<0.001$. The percent of the phenotypic variation explained (%PVE) by a given QTL was calculated from the regression of each marker/phenotype combination. The percent phenotypic change or additivity (%A) associated with the presence of a PN allele at a given locus was calculated as $2\times100[(AB-AA)/AA]$, where AA was the phenotypic mean for individuals homozygous for the LE allele at the most-significant marker for the locus and AB was the mean for heterozygous individuals. Because half of the individuals in each BC_2F_1 plot would be heterozygous for any fragment that was heterozygous in the BC_2 generation, the factor of 2 was included to obtain the estimate of %A. Multiple regression analysis was performed in StatView (SAS Institute Inc., Cary, N.C.).

Results

Marker segregation and the genetic map

A total of 152 RFLP markers were genotyped for the BC_2 population. Of these, 110 (72%) were segregating and could be mapped; the remaining 42 markers were fixed for LE alleles. Many of the markers fixed for LE alleles corresponded to the chromosomal regions for which marker-assisted selection was applied to remove the wild parent allele in the BC_1 population. Thus, the top half of chromosome 1 was fixed for LE alleles as a result of selection at the *S* locus (TG125). Marker-assisted selection at *fw2.2* and *fw11.3* resulted in fixation of the middle of chromosome 2 and the bottom half of chromosome 11. In addition, selection at the *Sp* locus on chromosome 6 resulted in fixation of the middle part of this chromosome for LE alleles. Three other regions of the genome encompassing more than one marker were also fixed for LE alleles: the bottom of chromosome 1, a bottom portion of chromosome 4 and the top of chromosome 7. Fixation of these regions cannot be explained by marker-assisted selection. Instead, it may be the result of genetic drift because the BC_1 population that gave rise to the BC_2 was very small.

The average number of heterozygotes per locus was 27% which was nearly identical to the expected value of 25% for a BC_2 population. A total of 31 markers (28%) showed significant ($P<0.001$) deviation from the expected frequency of heterozygotes. Most of these markers were concentrated on six chromosomes. Three regions showed severe skewing with fewer heterozygotes than expected: the bottom of chromosome 5 (TG 60 to CT138, three markers), the top of chromosome 6 (CT216 to TG178, two markers) and the top of chromosome 11 (TG497 to TG523, three markers). Three larger chromosomal regions exhibited segregation distortion with an excess of heterozygotes. The bottom half of chromosome 7 (TG217 to CT195, six markers) and the top half of chromosome 10 (TG230 to TG408, five markers) were moderately skewed while the top half of chromosome 12 (TG180C to CT211A, five markers) was very severely distorted. More than 90% of the population was heterozygous for three of the markers (TG180C, TG68, TG263) in this region.

The 110 mapped markers fell into 15 linkage groups, as markers from the tops and bottoms of chromosomes 2, 4 and 6 could not be linked at the LOD 3.0 threshold (Fig. 1). In all, 87 (79%) of the markers were considered to be framework markers as they were positioned with a ripple at $LOD\geq3.0$. All but one of the remaining markers mapped to the intervals between framework markers at $2.0\leq LOD<3.0$. TG587 on linkage group 4 did not link to the rest of the linkage group, therefore it was assigned to a separate linkage group. The map spanned approximately 703 cM, 55% of the genetic distance encompassed by the high-density tomato map (Tanksley et al. 1992). Coverage was primarily limited by the high percentage of non-segregating markers (28%) many of which (23 of 41 markers) corresponded to regions that were affected by marker-assisted selecton. With only one exception (TG581 on the bottom of chromosome 6), the marker order of the framework map agreed with the high-density map.

Trait correlations

For traits measured in more than one location, the strongest correlations across locations were observed for YLD and RDY ($r=0.72$ and 0.71, respectively) in IS and CA1, and the yield-derived trait, BRY ($r=0.61$), at the same locations (data not shown). FW/FSZ also showed significant ($P<0.05$) positive correlations ($r=0.31$ to 0.53) across locations as did ER ($r=0.47$), SSC ($r=0.19$ to 0.33) and EC ($r=0.23$ to 0.27). None of the other traits that were measured in more than one location (FS, IC, PCP, FIR, PUF and PGY) showed significant correlations across locations.

Within each location, significant correlations were also detected between many traits. However, only those that were highly significant ($P<0.001$), or were observed in more than one location, are described here. For traits

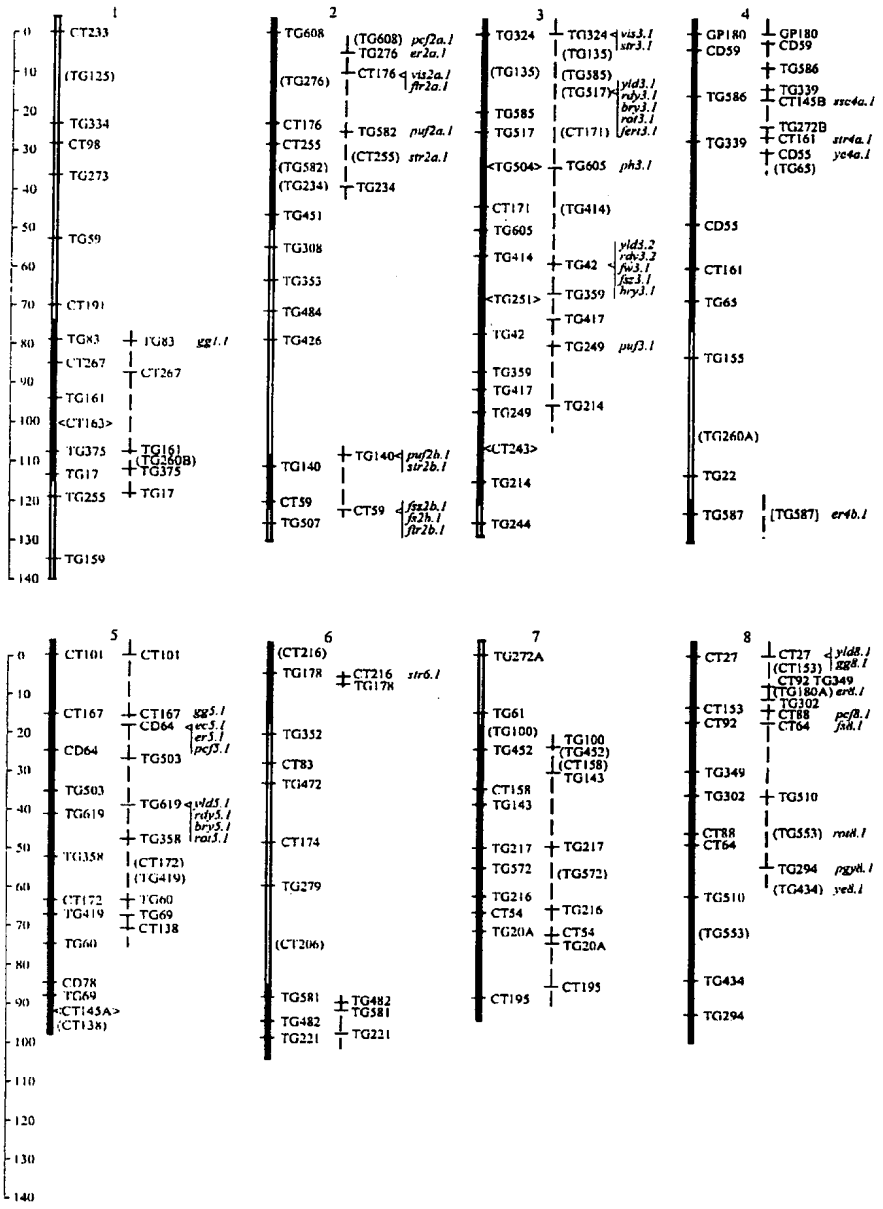
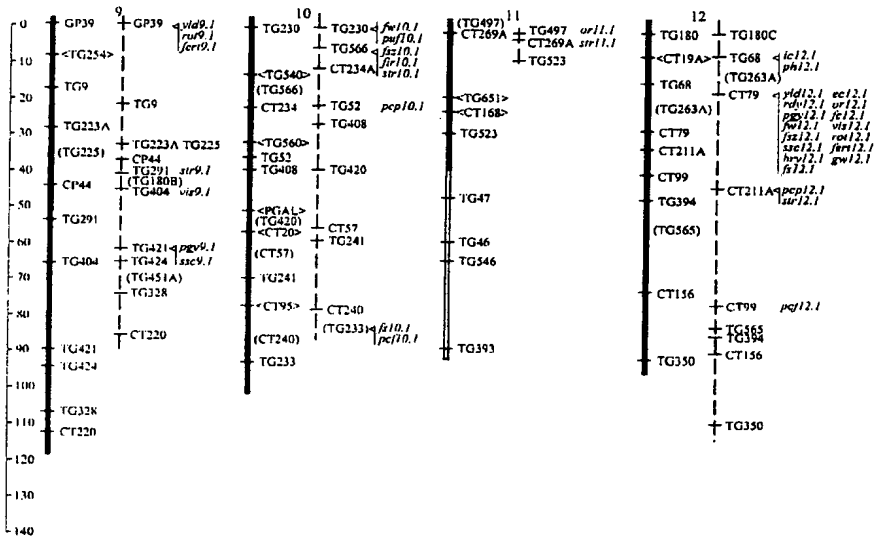


Fig. 1 Comparison of the BC_2 molecular linkage groups (*dashed lines*) to the corresponding chromosomes from the high-density tomato map (*bars*). The centiMorgan scale is given on the far left. Sections of the chromosomes drawn as *open bars* were not segregating for PN alleles in the population. Markers in "<>" were also fixed for LE alleles. Markers in"()" were ordered at LOD<3.0. The marker in "[]" did not link to the rest of the linkage group. Identified QTLs are shown to the right of the BC_2 linkage groups. Co-segregating QTLs are connected by a *vertical line* with a "<" to indicate the most-significant marker

measured in multiple locations, correlation values are averaged across locations. YLD and RDY were both positively correlated with FW (r=0.55 and 0.53, respectively) but were negatively associated with SSC (r=−0.46 and −0.45, respectively). Negative correlations were also observed between SSC and the traits FW/FSZ (r=−0.46), VIS (r=−0.68), EC (r=−0.23) and FERT (r=−0.28). FW/FSZ was positively correlated with EC in two locations (r=0.31), FERT in CA2 (r=0.40) and YE in CA1 (r=0.46). The fruit-color traits, EC and IC, were positively associated in all three locations (r=0.53).

Fig. 1 (continued)



QTLs detected for each trait

A total of 48 QTLs were identified for the 15 traits measured in IS, 43 (90%) of these were significant at $P<0.001$ and the remaining five were significant at $P<0.01$ and identified in at least one other location. A total of 39 QTLs were detected for the 18 traits measured in CA1 with 28 (72%) of the loci detected at $P<0.001$. In addition, 25 QTLs were identified for the 11 traits measured in CA2 with 24 (96%) of the loci identified at the more-stringent significance threshold. For many traits, the QTLs detected in different locations mapped to the same chromosomal positions. When these overlapping QTLs are counted as single loci, it is found that 84 different loci were identified for the 25 traits measured in the study. QTLs were detected on all chromosomes except chromosome 7 with the most loci found on chromosome 12, 20 QTLs. The QTL identified for each trait are described below, listed in Table 1 and mapped in Fig. 1.

Yield traits

Six QTL were identified for total yield on five different chromosomes. All six of these loci were detected in both IS and CA1. The locus on chromosome 12, *yld12.1*, was the most-significant and explained 56% and 32% of the phenotypic variation for the trait in CA1 and IS, respectively. For the other QTL, the percent phenotypic-variance explained (%PVE) was 10% or less. As determined by multiple regression analysis, together the six loci explained 39 and 28% of the variation for yield in CA1 and IS, respectively. The PN allele of only *yld9.1*, which was a relatively minor QTL in terms of significance and magnitude of effect, was associated with increased yield. Four QTLs were detected for red yield on three different chromosomes and all four were identified in both locations where this trait was measured. All of the QTLs had small %PVEs except for *rdy12.1* which controlled as much as 61% of the variance for red yield (in CA1). Overall, the four RDY loci accounted for 34% of the red yield variation in CA1. None of the QTLs showed favorable effects from the wild-alleles.

Three QTLs were found for the percent green yield on chromosomes 8, 9 and 12. Although the loci were highly significant ($P<0.0001$), none of them was detected in both IS and CA1, the two locations where the trait was measured. The most-significant QTL, *pgy12.1*, accounted for up to 19% of variation for the trait. For all three loci, the LE alleles were associated with an increased percent green yield. Three loci were also identified for fertility. As with YLD, RDY and PGY, the locus on chromosome 12, *fert12.1*, had the greatest magnitude of effect, a PVE of 41%. The three FERT loci accounted for 24% of the variation for the trait at CA2. For one of the QTLs, *fert 9.1*, the PN allele was associated with increased fertility. Five QTLs were detected for the amount of rotten fruit on the plants. Similar to the other yield traits, the locus on chromosome 12, *rot12.1*, was the most-significant and explained 19% of the variation in the trait. The other QTLs each accounted for less than 10% of the PVE. Together, the five ROT loci explained 24% of the variation for the amount of rot in IS. For all but one locus, *rot9.1*, the wild-alleles were associated with an increase in the amount of rotten fruit.

Table 1 Putative QTLs identified for each trait. *P*-values for the most-significant marker for each locus are given for Israel (IS) and the two California locations (CA1 and CA2). *Nd* indicates that the trait was not determined at that location, *ns* indicates that the marker was not significant. The percent phenotypic variation explained (%PVE) and percent additivity (%A) are only given for the location for which the QTL was most-significant (indicated by *P*-value in bold). The favorable-allele column indicates whether the *L. esculentum* (LE) or *L. pennellii* (PN) allele was associated with an agronomically favorable effect on the trait. The relative significance of each QTL is coded such that the number of +s indicates the number of locations at which the QTL was detected at $0.001<P<0.01$ and the number of *s indicates the number of locations at which the QTL was identified at $P<0.001$. Populations with putative orthologs are abbreviated: CA = *Capsicum annuum*, pepper; CM = *Lycopersicon cheesmanii*; H = *L. hirsutum*; PF = *L. parviflorum*; PM = *L. pimpinellifolium*; PN = *L. pennellii*; PV = *L. peruvianum*; SM = *Solanum melongena*, eggplant. CA1 = intra-specific *C. annuum* F_2 population (Ben Chaim et al. 2001), CA2 = *C. annuum* x *C. frutescens* advanced backcross population (Rao et al. 2003), CM1 = *L. esculentum* x *L. cheesmanii* F_2 population (Paterson et al. 1991); CM2 = *L. esculentum* x *L. cheesmanii* recombinant inbred population (Goldman et al. 1995); CM3 = *L. esculentum* x *L. cheesmanii* F_2 population (Monforte et al. 1997); H1 = *L. esculentum* x *L. hirsutum* advanced backcross population (Bernacchi et al. 1998); H2 = *L. esculentum* x *L. hirsutum* near-isogenic lines (Monforte et al. 2001); PF = *L. esculentum* x *L. parviflorum* advanced backcross population (Fulton et al. 2000); PM1 = *L. esculentum* x *L. pimpinellifolium* advanced backcross population (Tanksley et al. 1996); PM2 = *L. esculentum* x *L. pimpinellifolium* backcross population (Grandillo and Tanksley 1996); PM3 = *L. esculentum* x *L. pimpinellifolium* F_2 population (Monforte et al. 1997), PM4 = *L. esculentum* x *L. pimpinellifolium* backcross population (Chen et al. 1999); PM5 = *L. esculentum* x *L. pimpinellifolium* F_2 population (Lippman and Tanksley 2001); PM6 = *L. esculentum* x *L. pimpinellifolium* advanced backcross population (Fulton et al. 2002); PM7 = *L. esculentum* x *L. pimpinellifolium* inbredbackcross lines (Doganlar et al. 2002a); PN = *L. esculentum* x *L. pennellii* introgression lines (Eshed and Zamir 1995); PV1 = *L. esculentum* x *L. peruvianum* advanced backcrosspopulation (Fulton et al. 1997b); PV2 = *L. esculentum* x *L. peruvianum* advanced backcrosspopulation (Fulton et al. 2002); PV3 = *L. esculentum* x *L. peruvianum* near-isogenic lines (unpublished data); SM = *Solanum linnaeanum* x *S. melongena* F_2 population (Doganlar et al. 2002b)

Trait	QTL	Chr	Marker	*P*-value			%PVE	%A[a]	Favorable allele[b]	Relative significance	Populations with putative orthologs
				IS	CA1	CA2					
Total yield	*yld3.1*	3	TG517	**0.0003**	0.002	nd	8	−71	LE	*+	
	yld3.2	3	TG42	**0.0003**	0.002	nd	8	−77	LE	*+	H1;PF;PN
	yld5.1	5	TG619	**<0.0001**	0.0003	nd	10	−84	LE	**	
	yld8.1	8	CT27	**0.002**	0.004	nd	6	−78	LE	++	PF;PV1
	yld9.1	9	GP39	**0.005**	0.004	nd	5	86	PN	++	
	yld12.1	12	CT79	<0.0001	**<0.0001**	nd	56	−128	LE	**	H1;PV1
Red yield	*rdy3.1*	3	TG517	**0.0009**	0.005	nd	7	−70	LE	*+	
	rdy3.2	3	TG42	**0.0003**	0.005	nd	8	−80	LE	*+	
	rdy5.1	5	TG619	**0.0001**	0.001	nd	9	−84	LE	*+	PF;PV1
	rdy12.1	12	CT79	<0.0001	**<0.0001**	nd	61	−114	LE	**	H1
Percent green yield	*pgy8.1*	8	TG294	**0.0001**	ns	nd	9	271	LE	*	PV1
	pgy9.1	9	TG421	**<0.0001**	ns	nd	12	313	LE	*	H1
	pgy12.1	12	CT79	ns	**<0.0001**	nd	19	509	LE	*	
Fertility	*fert3.1*	3	TG517	nd	nd	**0.0002**	9	−53	LE	*	
	fert9.1	9	GP39	nd	nd	**0.0007**	7	72	PN	*	
	fert12.1	12	CT79	nd	nd	**<0.0001**	41	−97	LE	*	
Rotten	*rot3.1*	3	TG517	**0.0003**	nd	nd	8	−81	PN	*	
	rot5.1	5	TG619	**0.0003**	nd	nd	7	−81	PN	*	
	rot8.1	8	TG553	**0.0002**	nd	nd	9	−80	PN	*	PM7
	rot9.1	9	GP39	**0.0008**	nd	nd	7	121	LE	*	
	rot12.1	12	CT79	**<0.0001**	nd	nd	19	−106	PN	*	
Soluble solids content	*ssc4a.1*	4a	CT145b	ns	**0.0006**	ns	8	−15	LE	*	PF;PM1,6
	ssc9.1	9	TG421	ns	**0.0006**	ns	8	15	PN	*	H1;PF;PM7;PN
	ssc12.1	12	CT79	**<0.0001**	<0.0001	0.0005	30	48	PN	***	PM1,4;PN;PV2
Brix × red yield	*bry3.1*	3	TG517	**0.0003**	ns	nd	8	−65	LE	*	
	bry3.2	3	TG42	**0.0003**	0.006	nd	8	−68	LE	*+	H1;PN
	bry5.1	5	TG619	**<0.0001**	0.002	nd	11	−79	LE	*+	PF;PV1
	bry12.1	12	CT79	<0.0001	**<0.0001**	nd	53	−126	LE	**	H1;PN;PV1
Viscosity	*vis2a.1*	2a	CT176	nd	**0.0009**	nd	7	19	LE	*	
	vis3.1	3	TG324	nd	**0.0004**	nd	8	−20	PN	*	
	vis9.1	9	TG404	nd	**<0.0001**	nd	18	−24	PN	*	PM1;PV1
	vis12.1	12	CT79	nd	**<0.0001**	nd	18	−30	PN	*	
pH	*ph3.1*	3	TG605	nd	**0.0007**	nd	8	4	PN	*	
	ph12.1	12	TG68	nd	**<0.0001**	nd	18	7	PN	*	PM4;PV1
Pericarp thickness	*pcp10.1*	10	TG52	ns	ns	**<0.0001**	17	−24	LE	*	PF;PM2
	pcp12.1	12	CT211	**0.001**	ns	0.007	6	16	PN	++	
Fruit firmness	*fir2a.1*	2a	CT176	**<0.0001**	ns	ns	16	−50	LE	*	
	fir2b.1	2b	CT59	ns	ns	**<0.0001**	13	−44	LE	*	PM1
	fir10.1	10	TG566	ns	ns	**0.0006**	9	−31	LE	*	PF;PM7

Table 1 (continued)

Trait	QTL	Chr	Marker	*P*-value			%PVE	%A[a]	Favorable allele[b]	Relative significance	Populations with putative orthologs
				IS	CA1	CA2					
Stem retention	*str2a.1*	2a	CT255	**0.0001**	nd	nd	10	55	LE	*	
	str2b.1	2b	TG140	**<0.0001**	nd	nd	17	102	LE	*	H1;PV1
	str3.1	3	TG324	**<0.0001**	nd	nd	11	65	LE	*	
	str4a.1	4a	CT161	**<0.0001**	nd	nd	15	73	LE	*	
	str6a.1	6a	CT216	**0.0001**	nd	nd	9	69	LE	*	
	str9.1	9	TG291	**0.0002**	nd	nd	8	44	LE	*	H1;PV1
	str10.1	10	TG566	**0.0003**	nd	nd	8	42	LE	*	H1;PF;PM2
	str11.1	11	CT269	**<0.0001**	nd	nd	25	139	LE	*	
	str12.1	12	CT211	**0.0002**	nd	nd	8	−35	PN	*	
Fruit-weight	*fw3.1*	3	TG42	**0.0002**	0.004	nd	8	−26	LE	*+	CM1,2;PF;PM1,4,5; PN;CA1,2;SM
	fw10.1	10	TG230	**0.0002**	0.004	nd	8	−20	LE	*+	PF;PN;PV1
	fw12.1	12	CT79	**<0.0001**	<0.0001	nd	20	−30	LE	**	PM4;PV1
Fruit size	*fsz2b.1*	2b	CT59	nd	nd	**0.0006**	8	−24	LE	*	CM1,3;H1;PF; PM1,3,5;PN;PV;CA1,2
	fsz3.1	3	TG42	nd	nd	**0.0008**	8	−23	LE	*	CM1,2;PF;PM1,4,5; PN;CA1,2
	fsz10.1	10	TG566	nd	nd	**<0.0001**	18	−29	LE	*	PF;PN;PV1
	fsz12.1	12	CT79	nd	nd	**<0.0001**	16	−32	LE	*	PM4;PV1
Fruit shape	*fs2b.1*	2b	CT59	ns	0.001	**0.0006**	8	−16	LE	*+	PF;PM4;PV1
	fs8.1	8	CT64	**<0.0001**	0.0001	ns	12	−26	LE	**	H1;PF;PM2;PV1; CA1
	fs10.1	10	TG233	0.007	ns	**<0.0001**	17	−21	LE	*+	
	fs12.1	12	CT79	ns	ns	**0.0002**	10	−19	LE	*	PM4;PV1
Fruit external color	*ec5.1*	5	CD64	ns	ns	**0.0001**	12	−48	LE	*	H1
	ec12.1	12	CT79	<0.0001	0.0001	**<0.0001**	16	−44	LE	***	PF;PV1
Fruit internal color	*ic12.1*	12	TG68	**0.0002**	ns	ns	8	−48	LE	*	PF
Fruit orange color	*or11.1*	11	TG497	nd	nd	**0.0004**	10	86	LE	*	
	or12.1	12	CT79	nd	nd	**<0.0001**	20	142	LE	*	
Fruit color (lab)	*fc12.1*	12	CT79	nd	**<0.0001**	nd	18	−45	LE	*	PV1
Puffiness	*puf2a.1*	2a	TG582	0.009	**0.003**	nd	6	90	PN	++	
	puf2b.1	2b	TG140	**<0.0001**	ns	nd	10	47	PN	*	
	puf3.1	3	TG249	**0.0009**	ns	nd	7	25	PN	*	
	puf10.1	10	TG230	**0.0002**	ns	nd	8	24	PN	*	
Epidermal reticulation	*er2a.1*	2a	TG276	**0.0001**	nd	ns	9	87	LE	*	
	er4b.1	4b	TG587	**<0.0001**	nd	<0.0001	43	222	LE	**	H2;PF;PV3
	er5.1	5	CD64	0.002	nd	**0.0007**	9	47	LE	*+	
	er8.1	8	TG180a	**<0.0001**	nd	<0.0001	12	108	LE	**	
Percent cracked fruit	*pcf2a.1*	2a	TG608	nd	**<0.0001**	nd	10	133	LE	*	
	pcf5.1	5	CD64	nd	**0.0003**	nd	8	137	LE	*	
	pcf8.1	8	CT88	nd	**0.0009**	nd	7	118	LE	*	
	pcf10.1	10	TG233	nd	**<0.0001**	nd	13	144	LE	*	
	pcf12.1	12	CT99	nd	**0.0001**	nd	10	−84	PN	*	
Yellow eye	*ye4a.1*	4a	CD55	nd	**0.0006**	nd	11	87	LE	*	
	ye8.1	8	TG434	nd	**0.0001**	nd	13	−80	PN	*	
Grey wall	*gw12.1*	12	CT79	nd	**0.0004**	nd	10	−77	PN	*	
Green gel	*gg1.1*	1	TG83	nd	nd	**0.0003**	10	64	LE	*	
	gg5.1	5	CT167	nd	nd	**0.0001**	11	66	LE	*	
	gg8.1	8	CT27	nd	nd	**0.0001**	12	64	LE	*	PF

[a] %A=200(AB−AA)/AA where AA is the phenotypic mean for individuals homozygous for the *L. esculentum* allele at the most-significant marker and AB is the mean for heterozygous individuals

[b] For pH and fruit shape, this column indicates which allele was associated with an increase in the trait mean

Processing traits

The soluble-solids content of the fruit was determined in all three locations and three different loci were identified. Two relatively minor QTLs mapped to chromosomes 4 and 9, and the most-significant locus, *ssc12.1*, mapped to chromosome 12. This QTL accounted for up to 30% of the variation in soluble solids (in IS). Overall, the three loci accounted for 24% of the variation in the trait in CA1. The PN alleles for both *ssc9.1* and *ssc12.1* were associated with increased soluble solids. Four QTLs were detected for the derived-trait soluble solids (Brix) × red yield and were distibuted on three chromosomes: 3, 5 and 12. By far, the most-significant was *bry12.1* on chromosome 12 which explained 19% of the phenotypic variation in CA1 and 53% in IS. Together, the four BRY loci explained 23% of the Brix × red yield variation in IS. For all four loci, the LE alleles were associated with increased BRY.

The viscosity of juice from the tomatoes was measured only in CA1 where four QTLs were identified. Two of these loci, *vis9.1* and *vis12.1*, each explained 18% of the variation in juice viscosity and all together, the four QTLs accounted for 21% of the phenotypic variation. For all but *vis2a.1*, the wild-alleles had favorable effects and were associated with a more-viscous product. The pH of the fruit was also only determined in CA1. Two loci were detected, *ph3.1* and *ph12.1*, which accounted for 8 and 18% of the PVE for the trait, respectively. For both QTLs, the PN alleles were associated with increased acidity of the fruit.

Pericarp thickness was measured in all three locations and two different QTLs were identified. The more-significant locus was located on chromosome 10 and accounted for 17% of the variation for the trait. The combined effects of both loci explained 14% of the variation for pericarp thickness. The wild alleles for the two loci had opposite effects. The PN allele increased pericarp thickness for *pcp12.1* and decreased it for *pcp10.1*. Fruit firmness was also determined in all three locations and three loci were identified: *fir2a.1*, *fir2b.1* and *fir10.1*. *Fir2a.1* had the greatest %PVE, 16%. Together, the two loci identified in CA2 accounted for 15% of the variation in firmness. The LE alleles for all three QTLs were associated with firmer fruit. Stem retention was measured only in IS where nine QTLs were detected, the most for any trait in this study. These loci were distributed on eight different chromosomes with two QTLs on the separate linkage groups representing chromosome 2. Most of the loci had magnitudes of effect of 8 to 15%; however, the most-significant QTL, *str11.1*, explained 25% of the variation for stem retention. Overall, the nine loci explained 25% of the variation in the trait. With only one exception, *str12.1*, the LE alleles were associated with decreased stem retention.

Fruit appearance traits

Fruit size was assessed by weighing the fruit in IS and CA1 (FW), and with a visual scale in CA2 (FSZ). Three loci were detected for FW on chromosomes 3, 10 and 12. The QTL on chromosome 12, *fw12.1*, was the most-significant and explained as much as 20% of the PVE. Together, the three FW loci accounted for 12% of the variation for the trait in IS. Four QTLs were identified for FSZ, three of which corresponded closely to the FW loci. The fourth locus was identified on the lower portion of chromosome 2. The FSZ loci on chromosomes 10 and 12 were both highly significant and had similar magnitudes of effect, 18 and 16%, respectively. The combined effects of these four loci explained 15% of the phenotypic variation. It should be noted that marker-assisted selection was deliberately applied to remove three regions containing some of the most-significant fruit-weight QTLs previously identified in tomato: *fw1.1* near the *S* locus on chromosome 1, *fw2.2* on chromosome 2 and *fw11.3* on chromosome 11. Thus, the analysis for fruit-weight loci probably does not reflect the entire potential of this accession of *L. pennellii* as a source of the fruit-weight QTL. For all of the FW and FSZ loci, the PN alleles were associated with reduced fruit size as expected.

Fruit shape was controlled by four QTLs all of which were detected in two of the three locations where the trait was measured. *Fs8.1* had the highest significance levels; however, *fs10.1* had a larger effect on variation for fruit shape, maximums of 12 and 17%, respectively. The three loci detected in CA2 had a combined magnitude of effect of 18%. As expected based on the parental phenotypes, the PN alleles were associated with rounder fruit.

Fruit color was measured in four ways: external color (EC), internal color (IC), the amount of external orange color (OR) and a laboratory measurement on juice (FC). Two QTLs were identified for EC, *ec5.1* and *ec12.1*, accounting for 12 and 16% of the variation for the trait, respectively. Loci for IC were not identified in either CA location; however, one QTL was detected in IS, *ic12.1*. This locus only explained 8% of the phenotypic variation in internal fruit color. Two loci for OR were found in CA2. The more significant QTL, *or12.1*, had a magnitude of effect of 20%. Together, the two loci accounted for 16% of the variation for OR. Only one QTL was identified for FC, *fc12.1*, which explained 18% of the variation in the trait. For all of the fruit-color loci, the LE alleles were associated with improved, that is, redder color.

Puffiness or the amount of air space in the fruit locules was measured in two locations (IS and CA1) where four different QTLs were identified. Two of these QTLs mapped to the different linkage groups of chromosome 2 and the other two loci were located on chromsomes 3 and 10. All of the loci had relatively minor %PVEs of 10% or less and a combined magnitude of effect of 15%. The PN alleles were always associated with decreased puffiness. Epidermal reticulation describes the cantaloupe-like veining that is observed on the skin of some fruit. Four QTLs

controlling this trait were identified in IS and CA2 on chromosomes 2, 4, 5 and 8. The locus on chromsome 4, *cr4b.1*, was the most-significant and accounted for as much as 43% of the phenotypic variation in IS. In combination, the four loci had a PVE of 41%. For all four QTLs, the PN alleles were linked to increased reticulation. The percent of cracked fruit was only measured in CA1 where five QTLs were found. The most-significant of these was *pcf10.1* with a %PVE of 13% and, together, the loci accounted for 22% of the phenotypic variation for the trait. For only one QTL, *pcf12.1*, the wild alleles were associated with reduced cracking.

Yellow eye measured the penetration of the stem scar in the fruit. Two QTLs were identified for this trait, *ye4a.1* and *ye8.1*, controlling 11 and 13% of the phenotypic variation, respectively. The PN alleles for these loci had opposite effects increasing the percentage of fruit with yellow eye at *ye4.1* and decreasing it at *ye8.1*. Grey wall was measured only in CA1 where only one QTL was detected. This QTL, *gw12.1*, explained only 10% of the variation for the trait and its PN alleles were associated with improved fruit appearance. The color of the gel in cut fruit was assessed only in CA2. At this location, three QTLs were identified on chromosomes 1, 5 and 8, all of which had similar significances and magnitudes of effect ranging between 10 and 12%. None of the loci showed favorable effects from the wild parent-allele.

Discussion

Segregation distortion

A common feature of many interspecific plant populations is distorted segregation. This has been attributed to structural differences or loci that affect gamete transmission in the affected chromosomal regions (Zamir and Tadmor 1986). Six segments of the genome showed significant skewing of marker segregation ratios in the *L. esculentum* × *L. pennellii* BC_2 population. Regions on chromosomes 5, 6 and 11 had excesses of LE alleles while portions of chromosomes 7, 10 and 12 had higher than expected frequencies of PN alleles. The segregation distortion toward the LE genotype seen on chromosomes 6 and 11 was probably the result of the marker-assisted selection in these regions. Deviant segregation for some of these chromosomal regions has been reported in other tomato populations. For example, segregation distortion toward PN alleles of the top of chromosome 10 was observed in two *L. esculentum* × *L. pennellii* F_2 populations (deVicente and Tanksley 1993; Haanstra et al. 1999). Skewing was also detected in *L. hirsutum*, *L. peruvianum* and *L. parviflorum* interspecific populations for an overlapping region; however, in these populations excesses of LE alleles were observed (Bernacchi and Tanksley 1997; Fulton et al. 1997a, 2000). Similar to the current study, deviation from expected segregation ratios with an excess of LE alleles on chromosome 11 was reported in the *L. hirsutum*, *L. peruvianum* and *L. parviflorum* populations (Bernacchi and Tanksley 1997; Fulton et al. 1997a, 2000). It is interesting to note that none of these studies performed marker-assisted selection for this region. In contrast, deVicente and Tanksley (1993) observed that all of the markers on chromosome 11 were skewed toward the PN alleles in the *L. pennellii* F_2 population. The most-dramatic distortion observed in the BC_2 population occurred on a 45 cM portion of chromosome 12. Approximately 90% of the individuals in the population were heterozygous for this region. Zamir and Tadmor (1986) also saw a very marked preference for PN alleles in this region in an F_2 population.

The reasons for such dramatic segregation distortion are largely unknown. The BC_2 population was derived from a very small BC_1 population and therefore was very susceptible to genetic drift. Such drift might account for both fixation and segregation distortion in the population. Pelham (1968) attributed skewing on chromosome 9 of *L. peruvianum* to a gamete promoter gene. Preferential inheritance of the *L. peruvianum* allele in this region was also observed by Fulton et al. (1997a); however, conclusive evidence of a gamete promoter gene on the chromosome has not been reported. Analysis of the mechanism(s) responsible for distorted segregation is difficult as skewed regions vary greatly among species and even among populations derived from the same parent. Preferable inheritance of certain alleles in a given region has practical ramifications as it may necessitate additional backcross generations to achieve a desired level of homozygosity in breeding programs.

Correlations across locations and between traits

Correlations across locations were not significant for six of the 13 traits, measured in more than one location. However, there were strong associations across locations for the yield and yield-derived traits, and moderate correlations for fruit-weight, soluble solids and external color. From an agronomic perspective, these are the most-important traits for processing tomato cultivars. Similar to many previous studies, YLD/RYD and FW were found to be positively correlated (Stevens and Rudich 1978; Stevens 1986; Tanksley et al. 1996; Fulton et al. 1997b; Bernacchi et al. 1998; Fulton et al. 2000). However, both the yield and fruit-weight/size traits were negatively correlated with SSC. This is a well-documented phenomenon that suggests that attempted improvement in soluble solids will be at the expense of yield (Ibarbia and Lambeth 1971; Stevens 1986; Paterson et al. 1991; Tanksley et al. 1996; Fulton et al. 1997b; Bernacchi et al. 1998; Chen et al. 1999; Fulton et al. 2000; Doganlar et al. 2002a). A negative correlation was also identified between SSC and VIS, a result that was expected based on previous work (Stevens 1986, Fulton et al. 2000) and the fact that juice with higher soluble solids is, by its nature, more viscous. The significant positive correlation between FSZ and FERT suggests that an increased

number of fruit is not necessarily associated with a reduction in fruit size. External and internal fruit colors were also positively correlated as has been observed in *L. peruvianum*, *L. parviflorum* and *L. pimpinellifolium* mapping populations (Fulton et al. 1997b, 2000; Doganlar et al. 2002a).

Conservation of loci across environments

Of the 25 traits evaluated in this work, six were measured in all three locations, seven were assessed in two locations and 12 were determined at only one location. For the 13 traits that were evaluated in more than one environment, 43 QTLs were detected. Of these, two loci (5%) were identified at all three locations and 24 loci (56%) were identified at two locations. The only two QTLs identified in all three locations were *ssc12.1* and *ec12.1*. Notably, all of the YLD and RDY loci (six and four QTLs, respectively) were detected in both locations where these traits were measured. Moreover, all three FW loci and three of the four FS, BRY and ER QTLs were identified at two locations. This conservation across locations suggests that locus by environment interactions for these traits are relatively low. Strong conservation of QTLs across locations has been reported previously for several different interspecific AB-QTL tomato populations (Tanksley et al. 1996; Fulton et al. 1997b; Bernacchi et al. 1998; Fulton et al. 2000).

Co-localization of QTLs

The largest cluster of QTLs was on chromosome 12 where CT79 was a significant marker for 15 different loci. Smaller clusters of loci (three or more QTLs) were also present on chromosomes 2, 3, 5, 9 and 10. As expected, similar or related traits tended to be co-localized in the genome. For example, the four RDY QTLs always mapped with the YLD QTL. In addition, YLD loci mapped to the same regions as FERT QTLs on chromosomes 3, 9 and 12, and FW/FSZ QTLs on chromosomes 3 and 12. FW/FSZ loci also were co-localized with FS QTLs on chromosomes 2 and 12. Many of these clusters of related traits may reflect the pleiotropic effects of single loci. However, linkage of genes cannot be ruled out as a possible cause unless additional mapping is performed. For example, many studies have localized both fruit-weight and shape QTLs to the bottom half of chromosome 2 (reviewed in Grandillo et al. 1999). However, recent isolation of *fw2.2* (Frary et al. 2000) and *ovate* (Liu et al. 2002) have demonstrated that there are indeed distinct fruit-weight and shape-loci in this region of the genome.

QTLs with potential for breeding improved tomatoes

Many previous studies in tomato have demonstrated that phenotypically inferior wild species can be a source of agronomically favorable alleles (deVicente and Tanksley 1993; Eshed and Zamir 1995; Grandillo and Tanksley 1996; Tanksley et al. 1996; Fulton et al. 1997b; Bernacchi et al. 1998; Chen et al. 1999; Fulton et al. 2000; Doganlar et al. 2002a). In the present work, 11 (48%) of 23 traits had at least one QTL for which the *L. pennellii* allele had a positive agronomic effect. Traits for which effects were neither favorable nor unfavorable were excluded from this analysis. For example, pH was not included because increases or decreases in this character are not necessarily positive or negative but must be kept within an acceptable range for processing. Overall, 26% of the identified loci (20/78) had wild-alleles that enhanced the agronomic performance of the advanced backcross lines. Even higher percentages of traits with favorable wild-alleles were obtained with *L. peruvianum* (more than 50%, Fulton et al. 1997b), *L. hirsutum* (60%, Bernacchi et al. 1998) and *L. parviflorum* (70%, Fulton et al. 2000).

Some of the loci identified in this study may be targeted for breeding purposes. The *L. pennellii* allele(s) for the overlapping soluble solids and viscosity QTLs on chromosome 9 improved these two traits by 8 and 18%, respectively. Because of the related nature of these traits, it is probable that these effects are due to pleiotropy. The wild-alleles for several loci, centered around CT79 on chromosome 12, also had beneficial effects. The *L. pennellii* allele(s) at this location was (were) associated with a 48% increase in soluble solids content, an 18% improvement in viscosity, 19% and 10% reductions in fruit rot and cracking, respectively, a 16% increase in pericarp thickness and slight decreases in stem retention and grey wall. Unfortunately, cultivated alleles from the same region were also significantly linked to great improvements in total and red yields (56% and 61%, respectively), fertility (41%) and fruit-weight (20%), and lesser increases in external and internal fruit color.

Although it is possible that the multiple effects of this region of chromosome 12 are the result of pleiotropy, the diversity of phenotypes for the QTLs suggests that more than one locus does indeed exist in the neighborhood of CT79. Given the breeding potential of this region, it may be worthwhile to break the linkage between the sugar- and yield-traits so that the *L. pennellii* allele for improved soluble solids can be introgressed into cultivated tomato. This will require additional mapping to verify that the loci are indeed distinct and the screening of large populations for individuals that contain recombinations that break the linkages between the various traits. Such an approach has been used to break linkages between poor yield, low fruit-weight and high soluble solids in a *L. hirsutum* introgression (Monforte and Tanksley 2000), and between orange fruit color and high sugars in a *Lycopersicon chmielewskii* introgression (Frary et al. 2003a).

Loci shared among populations and species

With the addition of the present study, comprehensive QTL analyses are now available for AB populations derived from crosses with five different wild-species of tomato. In addition, the first QTL studies for pepper (Ben Chaim et al. 2001; Rao et al. 2003) and eggplant (Doganlar et al. 2002b; Frary et al. 2003b) have recently been published. This availability allows the identification of loci that are putatively conserved across tomato and its related wild- and crop-species. Of the 84 QTLs identified in this study, 38 (45%) are possibly the same as loci detected in other populations and species (Table 1). QTLs were considered to be potentially orthologous if they mapped to the same 20-cM region of the high-density tomato map (Tanksley et al. 1992). The majority (76%) of the putatively conserved loci were identified in three or more populations derived from different tomato species. In general, the yield-related, soluble solids and fruit size, shape and color traits had the highest proportions of QTLs that had been previously identified. This is probably because these traits have been examined in many studies whereas traits such as the amount of rotten and cracked fruit, puffiness and yellow eye have been examined in very few or no previous studies. The most-frequently identified loci were: *fsz2b.1*, detected in nine tomato populations representing six different species; *fw3.1/ fsz3.1*, identified in seven tomato populations encompassing four different species and *fs8.1*, detected in four tomato populations representing four different species. In addition, three loci appear to have orthologous counterparts outside of tomato. The fruit-weight/size QTL on chromosome 3 and the fruit-shape locus on chromosome 8 have been identified in pepper, *Capsicum annuum* (Ben Chaim et al. 2001; Rao et al. 2003). Moreover, *fsz2b.1* has been identified in both pepper and eggplant, *Solanum melongena* (Ben Chaim et al. 2001; Doganlar et al. 2002b; Rao et al. 2003). Such putative conservation of loci within the genus *Lycopersicon* and across other solanaceous species re-inforces the validity of the shared QTLs and supports the hypothesis that evolution and domestication in the Solanaceae has proceeded via mutations in loci that have been functionally conserved since divergence from a common ancestor (Doganlar et al. 2002b; Frary et al. 2003b).

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Appendix C

Report

of the

Tomato Genetics Cooperative

Number 54 – September 2004

University of Florida
Gulf Coast Research and Education Center
5007 60th Street East
Bradenton, FL 34203 USA

Foreword

The Tomato Genetics Cooperative, initiated in 1951, is a group of researchers who share an interest in tomato genetics, and who have organized informally for the purpose of exchanging information, germplasm, and genetic stocks. The Report of the Tomato Genetics Cooperative is published annually and contains reports of work in progress by members, announcements and updates on linkage maps and materials available. The research reports include work on diverse topics such as new traits or mutants isolated, new cultivars or germplasm developed, interspecific transfer of traits, studies of gene function or control or tissue culture. Relevant work on other Solanaceous species is encouraged as well.

Paid memberships currently stand at approximately 145 from 25 countries. Requests for membership (per year) US$15 to addresses in the US and US $20 if shipped to addresses outside of the United States--should be sent to Dr. J.W. Scott, jwsc@ifas.ufl.edu (see address information in Announcements section.) Please send only checks or money orders. Make checks payable to the **University of Florida.** We are sorry but we are **NOT** able to accept cash, wire transfers or credit cards.

Cover photo of Heinz 1706. Heinz 1706 is the tomato variety being sequenced in the worldwide tomato genome project. For further information see report by Rich Ozminkowski on p. 26 who provided the photo. Photo editing by John Petti.

- J.W. Scott

Revised List of Wild Species Stocks

Chetelat, R.T.

C.M. Rick Tomato Genetics Resource Ctr., Dept. Vegetable Crops, Univ. of California, Davis, CA 95616

The following list of 1,160 accessions of wild *Lycopersicon* and related *Solanum* species is a revision of the previous one, published in TGC vol. 51 (2001). Other types of TGRC stocks are catalogued in TGC 52 (monogenic mutants) and TGC 53 (miscellaneous genetic stocks). Inactive accessions have been dropped and new collections added to the present list. The new material includes populations of *L. chilense*, *L. peruvianum*, *S. lycopersicoides*, and *S. sitiens* collected in N. Chile in 2001, *L. hirsutum* and *L. pimpinellifolium* accessions donated by Miguel Holle, and *L. pimpinellifolium* donated by Fernando Nuez.

Seed samples of most accessions are available for distribution, upon request, for valid research purposes. Some accessions may be temporarily unavailable for distribution during regeneration. In general, only small quantities of seed can be provided – in most cases, 25 seed per accession for the self-pollinated species, 50 for the outcrossers, and 5-10 for the tomato-like *Solanum* spp. – and are intended to enable researchers to produce seed in larger quantities to satisfy their own needs. Accessions are grown for seed increase at UC-Davis, either in the field, for most of the selfing species, or in the greenhouse for the outcrossers. Accessions of the former are increased in small plots with as few as 6 plants, whereas the latter are regenerated from relatively large populations to maintain genetic variation.

For lack of space, only summary information on the collection site of each is presented here. To facilitate choice of accessions, those comprising the core subsets for each species are identified with an asterisk. More detailed passport information is available for each accession at our website (http://tgrc.ucdavis.edu). Geographic coordinates (lat/lon) have been estimated for most accessions collected from mainland S. America, and can be downloaded in several formats. Additional information will be provided upon request.

Acc. No.	Collection Site	Dept. / Prov.	Country
L. cheesmanii (39 accessions)			
LA0166*	Santa Cruz: Barranco, N of Punta	Galapagos Islands	Ecuador
LA0421*	San Cristobal: cliff E of Wreck Bay	Galapagos Islands	Ecuador
LA0422	San Cristobal: Wreck Bay	Galapagos Islands	Ecuador
LA0428	Santa Cruz: trail Bellavista to Miconia Zone	Galapagos Islands	Ecuador
LA0429*	Santa Cruz: crater in highlands	Galapagos Islands	Ecuador
LA0434	Santa Cruz: Rambech Trail	Galapagos Islands	Ecuador
LA0437	Isabela: ponds N of Villamil	Galapagos Islands	Ecuador
LA0521	Fernandina: inside Crater	Galapagos Islands	Ecuador
LA0522	Fernandina: outer slopes	Galapagos Islands	Ecuador
LA0524	Isabela: Punta Essex	Galapagos Islands	Ecuador
LA0528B	Santa Cruz: Academy Bay	Galapagos Islands	Ecuador
LA0529	Fernandina: crater	Galapagos Islands	Ecuador
LA0531*	Baltra: Barranco slope, N side	Galapagos Islands	Ecuador
LA0746*	Isabela: Punta Essex	Galapagos Islands	Ecuador
LA0749*	Fernandina: N side	Galapagos Islands	Ecuador
LA0927	Santa Cruz: Academy Bay	Galapagos Islands	Ecuador
LA0932	Isabela: Tagus Cove	Galapagos Islands	Ecuador

Acc. No.	Collection Site	Dept. / Prov.	Country
L. hirsutum cont'd			
LA0387	Santa Apolonia	Cajamarca	Peru
LA1033	Hacienda Taulis	Lambayeque	Peru
LA1295	Surco	Lima	Peru
LA1298	Yaso	Lima	Peru
LA1347*	Empalme Otusco	La Libertad	Peru
LA1352	Rupe	Cajamarca	Peru
LA1353*	Contumaza	Cajamarca	Peru
LA1354	Contumaza to Cascas	Cajamarca	Peru
LA1361*	Pariacoto	Ancash	Peru
LA1362	Chacchan	Ancash	Peru
LA1363*	Alta Fortaleza	Ancash	Peru
LA1366	Cajacay	Ancash	Peru
LA1378	Navan	Lima	Peru
LA1391	Bagua to Olmos	Cajamarca	Peru
LA1392	Huaraz to Casma	Ancash	Peru
LA1393	Caraz	Ancash	Peru
LA1557	Rio Huara	Lima	Peru
LA1559	Desvio Huamantanga	Lima	Peru
LA1560*	Matucana	Lima	Peru
LA1648	Above Yaso	Lima	Peru
LA1681	Mushka	Lima	Peru
LA1691	Yauyos	Lima	Peru
LA1695	Cacachuhuasin, Canete	Lima	Peru
LA1696	Huanchuy-Cacra	Lima	Peru
LA1717	Sopalache	Piura	Peru
LA1718	Huancabamba	Piura	Peru
LA1721*	Tlcrapo Viejo	Huancavelica	Peru
LA1731*	Rio San Juan	Huancavelica	Peru
LA1736	Pucutay	Piura	Peru
LA1737	Cashacoto	Piura	Peru
LA1738	Desfiladero	Piura	Peru
LA1739	W of Canchaque	Piura	Peru
LA1740*	W of Huancabamba	Piura	Peru
LA1741	Sondorillo	Piura	Peru
LA1753	Surco	Lima	Peru
LA1764	W of Canta	Lima	Peru
LA1772	W of Canta	Lima	Peru
LA1775	Rio Casma	Ancash	Peru
LA1777*	Rio Casma	Ancash	Peru
LA1778	Rio Casma	Ancash	Peru
LA1779	Rio Casma	Ancash	Peru
LA1918*	Llauta	Ica	Peru
LA1927	Ocobamba	Ica	Peru
LA1928*	Ocana	Ica	Peru
LA1978	Colca	Ancash	Peru
LA1980	Desvio Huambo	Ancash	Peru
LA2155*	Maydasbamba	Cajamarca	Peru
LA2156	Ingenio Montan	Cajamarca	Peru

(RIRDC) Rural Industries ...

Appendix D

The New Rural Industries

A handbook for Farmers and Investors
edited by Keith Hyde

Welcome to RIRDC's major publication with comprehensive information written by experts on nearly 100 new rural industries. To ensure speed of downloading, this html version does not contain any tables, photographs or figures. For the full version, download the pdf file from each page (you'll need the free Adobe Acrobat reader). Alternatively, you can buy the full, colour 570-page book from RIRDC ($40 plus $8 postage and handling) by phoning 02 6272 4819.

The Handbook was published in December 1997. Its usefulness has been enhanced by two RIRDC reports published since then:

a) 1988 - The New Rural Industries Financial Indicators (99/38) which provided information on the cost profile and possible returns of eight case studies; and

b) 2000 - Full Report: Financial Analysis of New Rural Industries – Volume II 00/133 HAS-6A (3.4 megs -)
Builds on Volume I (below) by widening coverage to other 'new industries'. Eleven enterprise options are analysed and benchmarked against two other options – wine grapes and Treasury Bonds. View a html version of the Executive Summary and Introduction. In addition to the report which you can download as a self-extracting zip file (3.4 megs), you can also download Excel files to help make site-specific analyses for an actual investment (Excel 97 or later required).

Excel files:

- Alpacca
- Durian
- Livestock Template
- Sesame
- South African Proteaceae
- Crop Template
- Emu
- Medicinal herbs
- Winegrapes
- Culinary Herbs
- Govt. Bonds
- Persimmon
- Dairy Goats
- Jojoba
- Red Deer

The Handbook

- Foreword
- Success factors for developing new rural industries - article
- Preface
- Marketing research for new industries - article

In Category Order

Animals

- Alpacas
- Cashmere
- The crocodile industry
- Dairy goats and goat milk products
- Deer farming
- Emu farming
- Goat meat

In Alphabetical order

- Abalone
- Acacias
- Adzuki
- Alpacas
- Banksias
- Barramundi
- Blackgram
- Blandfordia
- Boronias

21/08/03

http://www.rirdc.gov.au/pub/handbook/contents.html

- High prices for raw produce make it difficult to get good prices for value-added products. Many mainstream food manufacturers do not believe that a 'novelty', 'exotic' or 'clean and green' factor justifies higher prices than for conventional foods.
- Food manufacturers and consumers need to know that the food offered is safe to eat. This highlights the need for appropriate food-safety standards.
- Little is known about the most acceptable form of produce. Early market research suggests that there will be more demand for pureed, dried/ground, essences and flavours than for fresh or frozen whole fruits and nuts.

Varieties

There has so far been little genotype selection of improved plants, but the following species are at present the most commonly used and most in demand.

Bush tomato *(Solanum centrale)*. Also known as the desert raisin or in some Aboriginal communities as 'akudjura'. A small shrub with grey to green leaves; fruits turn from green to yellow when ripe and dry on the plant to resemble a raisin. It is intensely flavoured with a piquant, spicy taste and can be used as a spice or flavouring addition in most dishes where tomato is used.

Illawarra plum (*Podocarpus elatus*). Also known as brown pine. Evergreen conical tree, a member of the conifer family, which is sometimes used as a municipal street tree or in parks and gardens. Dark green leaves with flowers on both male and female trees; it has blue/black fruits (approx. 20 mm long—ripening during autumn/winter) with an inedible seed attached to the outside of the flesh at the opposite end to the stem. It has a subtle plum/pine flavour.

Kakadu plum (*Terminalia ferdinandiana*). Also known as billygoat, green or wild plum or murunga in East Arnhem land. A medium-sized deciduous tree with flower spikes in early summer followed by oval-shaped, green fruit with a large stone (ripening March-June). It has the world's highest fruit source of vitamin C.